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Sent: Monday, November 21, 2005 10:06 AM
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Ann Clin Biochem. 2000 Sep;37 (Pt 5):593-607.

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Review Article *Ann Clin Biochem* 2000; 37: 593–607

Genetic and biochemical markers for Alzheimer's disease: recent developments

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder of unknown aetiology, characterized by irreversible cognitive and physical deterioration. It is a major cause of morbidity and death in the elderly and a growing public health problem as life expectancy in the general population increases. AD is both genetically and phenotypically a heterogeneous disorder. An early-onset, familial type is recognized, as well as a later-onset, sporadic type. The diagnosis is made on clinical grounds, with the aid of a small number of additional investigations, using consensus criteria.[1] However, at autopsy about 10–20% of clinically diagnosed AD patients are found to have conditions other than AD. Therefore, genetic and/or biochemical markers that support the clinical diagnosis and can distinguish AD from cognitive symptoms attributable to ageing and from other dementias will be of great value. The identification of such accurate markers for the early diagnosis of AD is mandatory for the development of efficient pharmacological treatment, since therapy should be initiated at an early stage of the disease, before extensive and irreversible brain damage has occurred.

According to a recent consensus report,[2] the ideal biomarker for AD should fulfil the following criteria:

- detect a fundamental feature of the neuropathology
- be validated in neuropathologically confirmed cases
- have a sensitivity >85% for detecting AD
- have a specificity of 75–85% or more for distinguishing AD from other causes of dementia.

Moreover, a useful biomarker should be precise, reliable and inexpensive; it should be convenient to use and not harmful to the patient. An ideal biomarker would be helpful in confirming the diagnosis of AD in epidemiological screening, in predictive testing, in monitoring progression and response to treatment, and in studying brain–behaviour relationships.

The diagnostic utility as biomarkers of more than 60 substances in serum and cerebrospinal fluid (CSF), such as trace elements, metals, neurotransmitters, (neuro)peptides, proteins, amino acids and purines, has been reviewed by Basun[3] and by van Gool and Bolhuis.[4] None of the parameters described by these authors appeared to be useful to support the diagnosis of AD. Here, we review current hypotheses regarding the pathogenetic mechanisms in AD and describe new genetic and biochemical markers for the disease.

NEUROPATHOLOGICAL CHARACTERISTICS OF ALZHEIMER'S DISEASE

The neuropathological hallmarks of AD are senile (neuritic) plaques (SPs) and neurofibrillary tangles (NFTs). Several types of SPs can be distinguished, but all plaques contain extracellular deposits of amyloid-β peptide (Aβ) that include abundant amyloid fibrils with non-fibrillar forms. Aβ is generated during proteolytic processing of amyloid precursor protein (APP) (Fig. 1). For that reason, APP and its derivatives could provide good biological markers.

NFTs are intraneuronal lesions occurring in large numbers in the AD brain. The major components of NFTs are hyperphosphorylated, insoluble forms of tau protein, associated with microtubules. The insoluble tau aggregates in the NFTs are often conjugated with ubiquitin, a feature that is also found in other intraneuronal proteinaceous inclusions in aetiologically diverse disorders such as Parkinson's disease and diffuse Lewy-body disease. Furthermore, apolipoproteins E and J (ApoE and ApoJ), as well as

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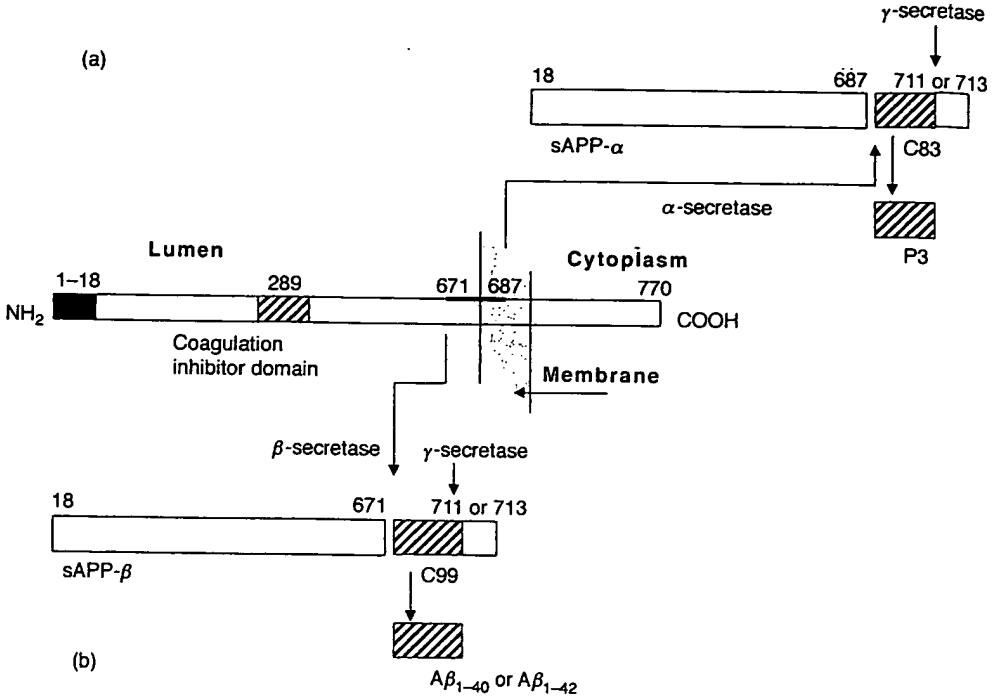

FIGURE 1. *Post-translational processing of amyloid precursor protein. The largest of the known APP alternate splice forms comprises 770 amino acids. A 17-amino acid residue signal peptide occurs at the N-terminus. Neurons express a more abundant 695-residue isoform, missing a 56-amino acid motif that is homologous to the Kunitz type of serine protease inhibitors. Proteolytic cleavage of APP by α-secretase after residue 687 enables secretion of soluble sAPP-α and retention of the C83 peptide in the membrane. The latter can undergo cleavage by γ-secretase after residues 711 or 713 to release the $P3_{1-40}$ and $P3_{1-42}$ peptides (a). Alternative proteolytic cleavage after residue 671 by β-secretase results in the secretion of the sAPP-β molecule and the retention of C99 peptide. The latter undergoes cleavage by γ-secretase at residues 711 or 713 to release the $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides (b).*

glycosylated acetylcholinesterase (AChE), protein S-100 and neuronal thread protein (NTP) have been demonstrated in the deposits in AD brain. Therefore, measurement of these parameters in CSF or other body fluids as diagnostic markers for AD would appear promising.

GENETIC VARIANTS OF ALZHEIMER'S DISEASE

AD is the most common form of dementia in the elderly, affecting 5–10% of the population aged over 65 years. It is well known that some Alzheimer patients have a family history of AD and a genetic predisposition to the disease. Estimates of the proportion of genetically based AD cases vary widely, but an established AD-related gene mutation occurs in less than 1% of all AD patients.

Mutations or polymorphisms in specific genes (presenilin-1, presenilin-2 and APP genes) have been found in early-onset familial AD. In contrast to deterministic genetic mutations, genetic factors may modify the risk of developing AD. Alleles of ApoE are the most powerful risk factors.

BIOMARKERS

Both genetic and biochemical markers for the different types of AD have been described in recent years (Table 1). The utility of these and other markers and of the risk factor ApoE genotype will be critically evaluated in this review.

Genetic markers

Amyloid precursor protein mutations

It has long been known that AD in certain families can occur in a familial form that transmits as an autosomal dominant trait (familial early-onset AD). A major step in understanding the pathological process leading to AD was the discovery that mutations in the

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TABLE 1. *Summary of useful genetic and biochemical markers for Alzheimer's disease*

Early-onset, familial type
• Genetic markers
– Presenilin-1 gene mutations
– Amyloid precursor protein gene mutations
– Presenilin-2 gene mutations
• Biochemical markers
– Plasma/CSF $A\beta_{1-42}$ peptide
– CSF tau protein
Late-onset, sporadic type
• Biochemical markers
– CSF $A\beta_{1-42}$ peptide
– CSF tau protein
– CSF AD7C-NTP

$A\beta_{1-42}$ peptide = amyloid β_{1-42} peptide; CSF = cerebrospinal fluid; NTP = neuronal thread protein.

APP gene on chromosome 21 are associated with the disease.[5–8] This finding is in accordance with the observation that individuals with trisomy 21 (Down's syndrome), who have a lifelong increase in APP expression, develop Alzheimer encephalopathy by their forties or fifties. Patients affected by Down's syndrome develop SPs containing amyloid depositions as early as the second or third decade of life, which may be the result of an increased gene dosage of APP. In 1991, a missense mutation in the APP gene was detected in early-onset AD,[6] and 1 year later a double mutation at the N-terminal of APP was found in an extended Swedish family.[5] All eight reported APP missense mutations linked to AD result in chronically elevated levels of a specific APP degradation product, the $A\beta_{1-42}$ peptide, in the brain. However, mutations in the APP gene are rare causes of AD, and only about 25 families have been found worldwide.[9] Nevertheless, these mutations have proved to be highly informative about the pathogenic mechanisms of AD in general. For example, transgenetic expression of mutant APP in mice provided the first reproducible animal models of AD.[10]

Presenilin mutations

A genetic linkage was found between early-onset Alzheimer families and chromosome 14.[11,12] The gene product presenilin-1 (PS1), is a previously unknown protein containing 467 amino acids. To date[9,13] at least 35 different mutations in the conserved domains of the protein have been identified in families with early-onset AD and this number is increasing rapidly.

Shortly after the discovery of PS1, another gene was identified that codes for presenilin-2 (PS2). The DNA sequence, on chromosome 1, was found to be 67% homologous to that encoding for PS1. Until now, two gene mutations of PS2 have been described in AD patients.[14,15] Finding a mutation in the PS1 or APP gene has a high predictive value for the development of AD. PS1 and PS2 are homologous, polytopic membrane proteins that have so far been localized in the endoplasmic reticulum (ER) and the Golgi apparatus in mammals. They are expressed in most cell types, including neurons. The presenilin holoproteins undergo endoproteolysis, generating stable N- and C-terminal fragments that associate into higher molecular mass complexes in Golgi-type vesicles.[16]

The most clearly identified functions of presenilins are in embryonic development[17] for the proper formation of the axial skeleton, normal neurogenesis and survival of progenitor cells and neurons in specific brain subregions.[18] Furthermore, PS1 regulates the intramembraneous proteolysis of APP, thereby promoting the formation of $A\beta$.[19] Different investigators have found that presenilin mutations increase γ-secretase cleavage of APP, so increasing the formation of the $A\beta$ peptides, particularly $A\beta_{1-42}$.[13,20,21]

Tau mutations

The tau gene has so far not been found to be a site of mutations in familial AD.

Apolipoprotein E polymorphism

An important chapter in AD concerns the role of ApoE isoforms in the disease. ApoE is a plasma protein involved in the transport of cholesterol. In the central nervous system (CNS), ApoE is produced by astrocytes and is implicated in growth and repair of the nervous system during development or after injury.

The ApoE gene is localized on chromosome 19 and presents three alleles (ε2, ε3 and ε4) determining ApoE polymorphism. Pericak-Vance *et al.*[22] described evidence for a linkage in late-onset familial AD. Analysis of ApoE alleles in AD patients and controls demonstrated a highly significant association of ApoE ε4 and late-onset familial AD.

Approximately 40–50% of all patients with AD carry the ε4 allele, compared with 15–25% of controls.[23,24] Individuals heterozygous for ε4 have a 3–4-fold increased risk of developing AD and in ε4 homozygotes there is a 6–8-fold increased risk. The disease-promoting effect of inheriting one or two ε4 alleles seems to involve enhanced aggregation and/or decreased clearance of $A\beta$.[25–28]

The ε4 allele is a risk factor for, but not an invariant cause of, AD. In a meta-analysis of 5930 patients who met the criteria for probable or definitive AD and 8607 controls, the ApoE ε4 allele was identified as a major risk factor in all ethnic groups studied (Caucasian, African-American, Hispanic and Japanese), across all ages between 40 and 90 years, and equally in men and women.[29]

To evaluate the usefulness of the ApoE genotype as an adjunct in the diagnosis of AD in persons with dementia, the data of clinical diagnoses and diagnoses obtained at autopsy in 2188 patients from 26 AD centres in the USA were pooled.[30] The clinical criteria for the diagnosis of AD were highly sensitive (93%), but the specificity was low (55%), resulting in a high false positive rate.

By contrast, the sensitivity and specificity of the ApoE ε4 allele were 65% and 68%, respectively. Thus, these test characteristics are not sufficient to allow the sole use of ApoE genotyping as a diagnostic test for AD, especially not in presymptomatic individuals, but should be reserved for demented patients.[31] However, sequential use of ApoE genotyping in patients who fulfil the clinical criteria for AD significantly improves the specificity of the clinical diagnosis, reducing the false positive rate but also decreasing the sensitivity.

A recently found genetic polymorphism in the transcriptional regulation region of ApoE may also be linked with AD risk.[32,33]

Biochemical markers

Table 1 summarizes useful biochemical markers. First we describe APP metabolism in relation to the working hypotheses on the pathological mechanisms in AD, as this information is essential in evaluating the different biochemical markers.

Amyloid precursor protein metabolism

APP is a membrane-bound protein encoded by a gene on chromosome 21. It comprises a group of ubiquitously expressed polypeptides whose heterogeneity arises from both alternative splicing and post-translational processing[34] (Fig. 1). Neurons express an isoform of 695 amino acid residues; non-neuronal cells throughout the body express 751/770 residue splice forms. The difference between these forms is the presence of an exon encoding for a motif of 56 amino acids, homologous to the Kunitz type of serine protease inhibitors. Functions that have been postulated for APP include inhibition of certain serine proteases, enhancement of cell–substrate adhesion, neurotropic and other growth-promoting effects, and neuroprotective properties.

APP is composed of a large extracellular domain at the N-terminal site, followed by a transmembrane domain and an intracellular domain of 47 amino acids at the C-terminus.

The biosynthetic pathway of APP begins in the endoplasmic reticulum (ER), as with all membrane proteins.[35] A putative role of presenilins is the regulation of the trafficking of APP in the constitutive secretory pathway leading to full maturation of the APP holoprotein in the trans-Golgi network.

Cleavage of the 770 amino acid residue after residue 687 by a protease designated α-secretase creates a large, soluble fragment (sAPP-α) that is released from the cell surface into the lumen of the vesicle and a membrane-retained C-terminal fragment of 83 amino acids (C83) (Fig. 1): In most cell types, a minority of all APP molecules undergoes α-secretase cleavage. An alternative cleavage by β- and γ-secretases leads to Aβ formation ($A\beta_{1-40}$ and $A\beta_{1-42}$). The proteolytic action of β-secretase on APP is essential for the generation of Aβ peptides. Recently β-secretase has been definitively identified by several groups.[36] β-Secretase is identical to the β-site APP-cleaving enzyme (BACE), also known as Asp-2. Most Aβ is extracellular, and only small amounts can be detected inside the cell. Aβ can be detected in plasma and CSF of humans and other mammals. Normally, the majority of Aβ released is $A\beta_{1-40}$. Only about 10% of Aβ extends to amino acid 42, $A\beta_{1-42}$.[9,37] The routing of APP through these pathways is under the control of phosphorylation, in particular by protein kinase C, a mechanism that is defective in sporadic AD fibroblasts. The $A\beta_{1-42}$ peptide aggregates far more rapidly into amyloid fibrils than does the $A\beta_{1-40}$ peptide.

Finally, the endosomal–lysosomal pathway is activated for the degradation of the C-terminal fragment left over by secretase processing and for the degradation of the full-length APP molecules recycling from the cell surface or derived directly from the biosynthetic pathway.

Working hypotheses on AD pathological mechanisms

The relationship between the observed lesions in the brain and the AD disease process has long been debated. Two broad hypotheses about the

mechanism have emerged
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brain. Overproduction of
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Amyloid precursor protei

The discovery of diseas
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Therefore, it seems rea
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AD.[51]

mechanism have emerged:[9,10,35] the amyloid cascade hypothesis and the inflammatory and neurotoxic cascade hypothesis. According to the amyloid cascade hypothesis, both familial and sporadic variants of AD are caused by amyloid accumulation, especially $A\beta_{1-42}$, in the brain. Overproduction of $A\beta_{1-42}$ or failure to clear this peptide leads to AD, primarily through amyloid deposition associated with cell death. Indeed, crossing mice transgenic for mutant APP with mice expressing a PS1 mutation results in a substantially accelerated AD-like phenotype with AD-like $A\beta_{1-42}$ plaques occurring early in life.[38] Moreover, the ability of presenilin mutations to selectively enhance $A\beta_{1-42}$ deposition in the brain has been directly demonstrated in AD patients carrying these mutations.[39,40] Evidence has recently emerged to support a direct involvement of presenilins in the γ-secretase cleavage of APP.[41]

According to the inflammatory and neurotoxic cascade theory, $A\beta_{1-42}$ accumulation and diffuse plaque formation is associated with local microglial activation, cytokine release, reactive astrocytosis and a multi-protein inflammatory response,[42–44] including the binding of the C1q component of the classical complement cascade by $A\beta$ and the triggering of this cascade. There is evidence that this $A\beta$-initiated inflammatory and neurotoxic process includes excessive generation of free radicals and peroxidative injury to protein and other macromolecules in neurons.[45,46] Moreover, altered ion homeostasis, in particular excessive calcium entry into neurons, may result in neuronal dysfunction and cell death.[47–49]

It is possible that $A\beta$ accumulation triggers the hyperphosphorylation of tau protein which precedes the assembly of these molecules into filaments. In a recent study, Schenk *et al.*[50] found that, in a mouse model of AD, immunization with $A\beta$ inhibits the formation of SPs and the associated dystrophic neurites, underlining the central role of $A\beta$ in developing AD. These results raise the possibility of future vaccination against human AD.

Amyloid precursor protein and metabolites

The discovery of disease-causing mutations in the APP gene has firmly established a key role for APP and $A\beta$ in the pathogenesis of AD. Therefore, it seems reasonable to detect and quantitate APP and its metabolites in plasma, serum or CSF and determine whether changes in concentration occur during development of AD.[51]

Amyloid precursor protein. Since APP is a membrane-bound protein, assays for APP in CSF measure the soluble or secreted derivative, generated by α/β-secretase cleavage. Henriksson *et al.*[52] reported markedly lower concentrations of APP in lumbar CSF of patients with AD compared with healthy controls, whereas ventricular CSF did not show any difference. Other studies did not discriminate in the site of collection of the CSF sample. In AD patients, substantially lower APP concentrations were found in some studies, while other studies showed relatively small reductions.[37,53–55] CSF APP does not seem to be a reliable biomarker for AD.

Amyloid-β peptide. The central pathological event in Alzheimer's disease is the deposition of $A\beta$ as amyloid fibrils within the SPs and cerebral blood vessels. It has been shown that $A\beta$ is a normal component of plasma and CSF.[56]

Another study[57] identified soluble $A\beta$ forms of 4, 3 and 3·7 kDa in AD brains but not in control brains free of amyloid deposits. This indicates that, in healthy brain, $A\beta$ is normally removed or bound to other proteins. Failure of this protective mechanism might cause amyloid formation and deposition in AD.

There is no significant difference in CSF total $A\beta$ peptide concentrations between AD patients, healthy controls and neurological controls, and therefore measuring total $A\beta$ ($A\beta_{1-40}+A\beta_{1-42}$) has no clear diagnostic utility.[37,58–60] However, Nitsch *et al.*[61] as well as Hock *et al.*[37] showed that CSF $A\beta$ concentrations were inversely correlated with a functional measure of dementia severity, indicating that determination of CSF $A\beta$ can be used to monitor the course of the disease in an individual patient.

$A\beta_{1-40}$ and $A\beta_{1-42}$ peptides. In the cortex of two familial AD patients with an APP mutation, a remarkable predominance of $A\beta_{1-42}$-positive over $A\beta_{1-40}$-negative plaques was found.[62] Diffuse plaques, representing the earliest stage of $A\beta$ deposition, were exclusively positive for $A\beta_{1-42}$, but completely negative for $A\beta_{1-40}$. Further, during the disease, aggregation of the more soluble $A\beta_{1-40}$ into fibrils can occur.[63]

As shown in Table 2, several studies[64–69] have reported no difference in the CSF $A\beta_{1-40}$ concentration between AD patients, healthy controls and neurological controls. However, decreased levels of $A\beta_{1-40}$ were reported in early- and mid-stage AD in one study.[66] In contrast,

TABLE 2. *Summary of the concentration of amyloid β_{1-40} ($A\beta_{1-40}$) and amyloid β_{1-42} ($A\beta_{1-42}$) in cerebrospinal fluid of Alzheimer's disease (AD) patients, healthy controls and neurological controls (NC) or non-Alzheimer-type dementia (DNAT)*

Ref.	Peptide	AD (ng/L)	*n*	Controls (ng/L)	*n*	NC/DNAT (ng/L)	*n*
64	$A\beta_{1-42}$	383 (76)	37	632 (156)	20	553 (177)	32
65	$A\beta_{1-40}$	2650 (1250)	24	3040 (1290)	11	2140 (770)	14
	$A\beta_{1-42}$	277 (105)	24	501 (266)	11	282 (86)	
68	$A\beta_{1-42}$	833 (379)	82	1485 (473)	60	1129 (464)	74
69	$A\beta_{1-42}$	Median 487 Range 394–622	150	Median 849 Range 682–1063	100	Median 603 Range 430–744	259
		(pmol/L)		(pmol/L)		(pmol/L)	
66	$A\beta_{1-40}$	1922 (547)	80	2311 (546)	24	1895 (662)	15
	$A\beta_{1-42}$	119 (63)	80	74 (30)	24	180 (95)	
67	$A\beta_{1-40}$	1498 (828)	93	1361 (859)	41	1495 (847)	89
	$A\beta_{1-42}$	110 (73)	93	242 (180)	41	232 (179)	

Data are expressed as mean (standard deviation) unless otherwise stated. *n* = number of patients.

many studies have shown that concentrations of CSF $A\beta_{1-42}$ are significantly reduced in patients with AD compared with age-matched normal subjects or patients with neurological disease.

Presumably, concentrations of soluble $A\beta_{1-42}$ in brain interstitial fluid decrease as the peptide becomes increasingly insoluble and forms deposits in the form of large numbers of diffuse and senile plaques. The drop in soluble $A\beta_{1-42}$ concentration in brain is reflected by a decline in the concentration of soluble peptide in CSF. CSF $A\beta_{1-42}$ seems to be a good biomarker for AD.

In a limited number of studies, the mean concentration of plasma $A\beta_{1-42}$ was reported to be consistently and significantly increased in subjects with each type of mutated gene known to be related to early-onset familial AD compared with age-matched controls.[13] However, the mean plasma $A\beta_{1-42}$ concentration was not increased in subjects with late-onset sporadic AD. At present, there are insufficient data on plasma $A\beta_{1-42}$ concentrations to allow any firm conclusions about the potential diagnostic utility of measuring plasma $A\beta_{1-42}$ in late-onset AD.

Studies are in progress on the diagnostic utility of detecting $A\beta$ peptides in human urine, but no clear conclusion is yet available.

Tau protein

Tau protein is a human brain phosphoprotein that binds to microtubules in the neuronal axons, thereby promoting microtubule assembly and stability. Multiple tau isoforms are produced from a single gene through alternative mRNA splicing. In adult human brain six isoforms are found, ranging from 352 to 441 amino acids.

The NFTs found in brains of AD patients are neuronal inclusions consisting of paired helical filaments (PHF), of which the main protein components are the six hyperphosphorylated tau proteins. The increased phosphorylation causes lack of binding to microtubules and is believed to be responsible for self-assembly into PHF. Current evidence suggests that protein kinases or protein phosphatases are involved in the abnormal hyperphosphorylation of tau.[70]

It has been demonstrated that CSF tau levels increase in the early stage of AD,[71–74] with the highest concentrations found in the mid-stage of the disease.[75] These results suggest that increases in tau can be detected even in patients with very mild impairment and short duration of symptoms. It is unclear whether the elevation of CSF tau is a result of dying neurons, dystrophic neurites or the generation of NFTs.

Using enzyme-linked immunosorbent assays (ELISAs), several studies[35,64,67,71,72,76–83] have shown that the concentrations of CSF tau are significantly elevated in AD patients compared with normal elderly control subjects (Table 3). However, elevated levels of CSF tau were also detected in patients with other dementias and acute or chronic neurological diseases.[79,83–89]

Therefore, the value of the CSF tau in discriminating AD from other neurological diseases may be limited. In addition to its potential as a diagnostic aid, simultaneous measurement of $A\beta_{1-42}$ and tau in the same CSF sample may become useful as a predictor of the progression to

TABLE 3. *Cerebrospinal f controls with neurological*

Ref.	AD
76	10·9 (4·9
35	361 (166)
77	1430 (739)
64	407 (241)
71	509 (255)
78	820 (90)
79	77·2 (45
80	722 (76)
81	279 (100)
82	251 (36)
72	524 (351)
67	489 (298)
83	796 (382)

Results are given in ng/L

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In post-mortem CS
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(n=121) was higher
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TABLE 3. *Cerebrospinal fluid tau protein concentrations of Alzheimer's disease (AD) patients, healthy controls and controls with neurological diseases (NC) or non-Alzheimer-type dementia (DNAT) as reported in different studies*

Ref.	AD	*n*	Controls	*n*	NC/DNAT	*n*
76	10·9 (4·9)	27	0·1 (0·5)	51	3·9 (7·4)	129
35	361 (166)	71	190 (80)	26	235 (104)	25
77	1430 (739)	24	816 (355)	14	790 (579)	26
64	407 (241)	37	212 (102)	20	168 (63)	32
71	509 (255)	36	177 (82)	14	163 (58)	19
78	820 (90)	14	380 (120)	36	<600	12
79	77·2 (45·5)	70	9·0 (4·5)	19	27·8 (38·7)	106
80	722 (76)	39	173 (12)	30	320 (40)	12
81	279 (100)	23	26 (11)	36	88 (61)	23
82	251 (36)	31	51 (7)	30	126 (104)	39
72	524 (351)	81	293 (140)	33	403 (248)	40
67	489 (298)	93	217 (128)	41	220 (113)	89
83	796 (382)	43	190 (157)	18	357 (193)	18

Results are given in ng/L and expressed as mean (standard deviation). *n* = number of patients.

AD in individuals with memory impairment who do not meet clinical criteria for dementia. Detecting elevated concentrations of tau in CSF is a promising ante-mortem marker for AD, and might possibly be useful for monitoring disease progression and response to treatment.[2]

The development of assays incorporating more specific anti-tau antibodies that can distinguish normally and abnormally phosphorylated tau may enhance the discriminative power of CSF tau assays.[90,91]

Neuronal thread proteins

Neuronal thread proteins (NTPs) are a family of molecules that are expressed in brain and are immunologically related to pancreatic thread protein. They are normally present in neurons and are found in large amounts in association with NFTs.[92] There are at least six NTP immunoreactive species. Increased CNS expression of a 41-kDa NTP is correlated with dementia in AD.[93] The gene coding for this NTP has been cloned and sequenced, and the protein (produced by recombinant techniques) has been used to generate both monoclonal and polyclonal antibodies to NTP (AD7C-NTP). Ghanbari and Ghanbari[94] developed an ELISA with a monoclonal antibody as catcher and polyclonal antibodies for detection.

In post-mortem CSF, the mean concentration of AD7C-NTP in cases of confirmed AD ($n=121$) was higher than in 19 age-matched control cases. In CSF from individuals with early possible or probable AD ($n=89$), AD7C-NTP was also elevated in comparison to the concentrations in CSF from 18 age-matched controls. The concentrations in AD patients were also elevated in comparison to controls with other neurological diseases ($n=41$; specificity = 98%) and ambulant patients with Parkinson's disease ($n=32$; specificity = 84%).[95] Therefore, NTP in CSF appears to be a promising marker for the diagnosis of AD (Table 1). Additional studies are required to establish the exact sensitivity and specificity of this marker.

Apolipoprotein E

Since ApoE is associated with Aβ in SPs, several groups have measured ApoE in CSF, in addition to ApoE genotyping.[96] Plasma ApoE seems to have limited ability to cross the blood–brain barrier and ApoE in CSF is essentially derived from the brain. Therefore, the CSF concentration may reflect cerebral ApoE production. ApoE is known to have a general function in brain repair. After injury, ApoE is produced and secreted by astrocytes, to scavenge cholesterol and other membrane lipids from degenerating axons and myelin sheets. At the time of sprouting and remyelinization, neuronal growth cones take up and re-use the lipids in membrane and myelin synthesis. This process of membrane lipid re-utilization may be an important repair mechanism in various degenerative brain disorders, and impairment of this mechanism might contribute to earlier presentation of degenerative disorders. Increased re-utilization of ApoE–lipid complexes in the brain may explain the lower concentration of CSF ApoE in AD.[97] However, ApoE has also been found to bind to Aβ *in vitro*, and to be adsorbed onto the Aβ deposits in

SPs.[98] Moreover, ApoE binds tau protein, the principal component of NFTs. Yamada *et al.*[99] found increased mRNA levels encoding for ApoE in AD brain, which suggests increased ApoE production.

Various studies reported significantly reduced CSF ApoE concentrations in AD patients compared with healthy controls, irrespective of ApoE genotype. However, other investigators found no difference or a significant increase.[97,100–105] The control patients with neurological disease generally demonstrated similar concentrations to those of the AD patients, thus limiting the usefulness of ApoE quantitation.

Melanotransferrin

In several neurodegenerative diseases, including AD and Parkinson's disease, large quantities of iron are deposited in the CNS. Iron depositions can directly harm tissues by catalysing the generation of oxygen radicals. Jefferies *et al.*[106] have identified a novel pathway of iron uptake into mammalian cells, independent of the transferrin receptor. The melanotransferrin molecule, also known as p97 protein, is essential for this pathway. In brain tissue derived from AD patients, melanotransferrin was detected in a subset of reactive microglia associated with SPs, indicating that iron uptake through this alternative pathway plays a role in AD.

Kennard *et al.*[107] have shown that p97 concentrations [mean (standard deviation)] are consistently elevated in the serum of AD patients [43·8 (11·6) ng/mL; $n=17$] compared with controls [7·0 (3·3) ng/mL; $n=15$]. There was no overlap between the groups, and the correlation between age and p97 serum concentration was not significant. However, a significant correlation was found between disease progression and increased p97 serum concentrations. Extrapolation of these data suggests that the p97 concentration may begin to increase about 2 years before the first clinical symptoms of AD. Quantitation of melanotransferrin in serum is a promising candidate biomarker for AD.

Apolipoprotein J

ApoJ (clusterin) is a lipoprotein present in most, if not all, physiological fluids, in particular in plasma and CSF.[108–110] In normal CSF, ApoJ appears to be complexed with Aβ,[111] in particular with Aβ_{1-40}. Using a well-characterized *in situ* perfused guinea pig brain model, it was demonstrated that ApoJ may facilitate receptor-mediated transport of Aβ_{1-40}-ApoJ complexes across the blood–brain barrier and the blood–CSF barrier.[112]

ApoJ is overexpressed in response to neuronal injury. Available information supports the view that its basic function is maintenance of membrane integrity in an environment that exposes cells to membrane-destabilizing factors that occur normally or are generated in abnormal, degenerative situations.[109] ApoE and ApoJ may have complementary functions in lipid homeostasis during CNS degeneration and synaptic remodelling. However, the roles may be opposite with respect to Aβ deposition in SPs, in which ApoE acts as a pathological chaperone, whereas ApoJ would be functioning as a physiological chaperone of Aβ.[113] ApoJ concentrations in the hippocampus of AD subjects were 60% higher ($P<0·001$) than in non-demented controls.[114] In post-mortem human brains, ApoJ concentrations have been shown to be about 40% higher in AD patients than in controls.[115]

As elevation of ApoJ may be indicative of tissue injury, it will be important to measure its concentrations in CSF. To date, no method for measuring ApoJ in CSF has been published, but Jenkins *et al.*[110] presented a competitive ELISA for plasma ApoJ. It might be possible to modify this method for quantitation of ApoJ in CSF.

S-100 protein

S-100 is located in the cytoplasm and nuclei of cells that express it, as well as in synaptosomes and synaptic membranes. S-100 is a dimer comprising two separate subunits, α and β. The subunit S-100-β has been implicated in development and maintenance of the nervous system and may also play a role in neuropathology because of its specific localization and selective overexpression in AD.

CSF concentrations of S-100 protein in AD, and also in patients with frontotemporal dementia and other non-neurological conditions, were found to be significantly increased in comparison with healthy controls,[116] pointing to a lack of specificity of S-100. Nevertheless, further studies on the value of serum and CSF S-100 concentrations in the diagnosis of AD seem to be justified.

Glycosylated acetylcholinesterase

There is no consistent change in acetylcholinesterase (AChE) activity in the CSF of AD patients, and the large overlap with controls prevents the use of AChE a for AD.

As AChE in the AD br lated, the hydrophobic pr AChE may serve as a seed senile plaques.[117] Lectin-bi AChE of AD patients difference in glycosylation with controls. Preliminar glycosylated CSF AChE marker for AD, with a sen and a specificity of 97%.[11

Combination of markers

The ApoE genotype doe total Aβ concentration, as Aβ are found in AD pati ε3/4 and ε4/4 alleles.[61] Ho demonstrated a negative CSF Aβ_{1-42} levels and th alleles.

It has been reported t trations of CSF tau prote ApoE genotype,[79] as well and clinical stage. Ho dies,[119,120] CSF tau conce be related to ApoE ge carrying the ApoE ε4 allel CSF tau concentrations t out the ε4 allele, and the tau was found in patient The ApoE genotype sh interpreting CSF tau co nation of the ApoE gen specificity and sensitivity nosis.

Combined analysis of A is interesting. In a plot o high tau/low Aβ_{1-42} qua dictive for AD, whereas t quadrant contained cont was found that patients and high CSF tau have having AD (22/23 = 96% who exhibit low tau an free of AD (28/28 = 100%

These results have Mulder *et al.* (unpubli controls (18/20) and no p low tau/high Aβ_{1-42}, and 18 AD patients showe values. Similar results Japanese multicentre stu improved in a longitudin

prevents the use of AChE as a diagnostic marker for AD.

As AChE in the AD brain is highly glycosylated, the hydrophobic property of anomalous AChE may serve as a seed for amyloid fibrils in senile plaques.[117] Lectin-binding analysis of CSF AChE of AD patients showed a significant difference in glycosylation ($P<0{\cdot}01$) compared with controls. Preliminary results suggest that glycosylated CSF AChE may be a diagnostic marker for AD, with a sensitivity as high as 80% and a specificity of 97%.[118]

Combination of markers

The ApoE genotype does not influence CSF total Aβ concentration, as similar values of CSF Aβ are found in AD patients with ApoE ε3/3, ε3/4 and ε4/4 alleles.[61] However, Galasko *et al.*[68] demonstrated a negative correlation between CSF Aβ_{1-42} levels and the number of ApoE ε4 alleles.

It has been reported that increased concentrations of CSF tau protein are independent of ApoE genotype,[79] as well as age of disease onset and clinical stage. However, in other studies,[119,120] CSF tau concentration was found to be related to ApoE genotype. AD patients carrying the ApoE ε4 allele demonstrated higher CSF tau concentrations than AD patients without the ε4 allele, and the highest value of CSF tau was found in patients with two ε4 alleles. The ApoE genotype should be considered in interpreting CSF tau concentrations. Determination of the ApoE genotype can increase the specificity and sensitivity of the clinical diagnosis.

Combined analysis of Aβ_{1-42} and tau in CSF[64] is interesting. In a plot of tau versus Aβ_{1-42}, the high tau/low Aβ_{1-42} quadrant was highly predictive for AD, whereas the low tau/high Aβ_{1-42} quadrant contained control individuals only. It was found that patients with low CSF Aβ_{1-42} and high CSF tau have a strong likelihood of having AD (22/23 = 96%). Conversely, patients who exhibit low tau and elevated Aβ_{1-42} were free of AD (28/28 = 100%).

These results have been reproduced by Mulder *et al.* (unpublished data). Eighteen controls (18/20) and no patients (0/20) exhibited low tau/high Aβ_{1-42}, and one control as well as 18 AD patients showed high tau/low Aβ_{1-42} values. Similar results were obtained in a Japanese multicentre study,[67] and the sensitivity improved in a longitudinal evaluation.

Galasko *et al.*[68] studied 82 patients with probable AD, including 24 with very mild dementia [Mini-Mental State Examination (MMSE) score >23/30], 60 normal elderly control subjects and 74 subjects with neurological disorders. High CSF tau and low CSF Aβ_{1-42} levels discriminated AD patients from elderly controls. However, in subjects in the neurological control group with a CSF protein profile suggesting AD, follow-up at autopsy would be required to decide whether the CSF results are false positives or whether AD is the primary or a concomitant cause of dementia. The multicentre study of Hulstaert *et al.*[69] also confirmed the value of a combined determination of markers.

We conclude that the combined analyses of CSF Aβ_{1-42} and CSF tau can discriminate AD patients from normal elderly control subjects, supporting the use of these parameters to distinguish early AD from ageing.

To discriminate AD from other forms of dementia, especially vascular and frontotemporal dementia, further studies are needed to develop more sensitive methods for current and potential markers.

Other potential markers

AD involves profound biochemical and molecular alterations in the CNS. Increased phosphorylation of tau and other cytoskeletal proteins has been demonstrated in neurons,[121] as well as aberrant expression of genes modulated with neuritic sprouting such as the growth-associated protein GAP-43,[122] constitutive endothelial nitric oxide synthase,[123] transforming growth factor-β[124] and metallothionein-3.[125] An increased expression of genes associated with glial cell activation, such as glial fibrillary acidic protein,[126] and α_1-antichymotrypsin[127] is also found. Moreover, there are alterations in expression of genes coding for proteins protecting neurons from either cytotoxic or programmed cell death, including glycoprotein-2,[128] cathepsin D,[129] superoxide dismutase 1,[130] mitochondrial cytochrome oxidase,[131] C1q component of complement,[132] Calbindin D28k[133] and Bcl-2.[134]

Advanced glycation end-products (AGEs) may play an important role in the pathogenesis of AD. These are mainly found in NFTs and in about 5% of the SPs. However, SPs and NFTs were also AGE-positive in non-Alzheimer neurodegenerative diseases.[135] All these alterations in the CNS may lead to changes in CSF concentrations of AGEs.

Another recent finding is the presence of molecular misreading mutant forms of APP and ubiquitin in SPs and NFTs in AD brains.[136] In this study, mutant APP was detected in 15/21 AD patients, while aberrant ubiquitin was present in all patients. CSF ubiquitin is largely of intrathecal origin, which indicates that mutant ubiquitin may also be present in CSF and might be a sensitive biomarker for AD.[137]

CONCLUSIONS

Our present knowledge of the aetiology of AD mainly focuses on the amyloid cascade hypothesis. The genetic and the sporadic variants may have a common pathophysiology, where disturbances in APP metabolism occur as an early event.

Genetic markers such as PS1, PS2 and APP have been found to be reliable markers in diagnosing familial AD. A large polymorphism in the gene encoding for α_2-macroglobulin has been associated with an increased risk for late-onset AD.[138]

It can be predicted that, within a decade, a sizeable number of additional genes will be implicated, most of them probably acting as polymorphic risk factors in some populations.[10] There is consensus that searching for PS1, PS2 and APP genetic markers should be limited to probands and families with a pattern of early-onset AD, in a strict research setting.

Concentrations of CSF $A\beta_{1-42}$ are significantly reduced in patients with familial AD in comparison to controls with neurological disease and normal subjects. In sporadic AD, significantly decreased levels of CSF $A\beta_{1-42}$ are found in many patients, but there is some overlap with the control groups.

Measuring CSF $A\beta_{1-42}$ in conjunction with other parameters, particularly CSF tau protein and ApoE genotype, could be potentially useful for supporting early diagnosis of AD. Commercial kits are available for quantitation of tau protein and $A\beta_{1-42}$ as well as determination of the ApoE genotype.

AD7C-NTP is elevated in CSF samples from individuals with early possible or probable AD, in comparison to the CSF concentrations of age-matched controls. The concentrations in AD patients were higher than those in controls with neurological disease and ambulant patients with Parkinson's disease. Therefore, CSF AD7C-NTP appears to be a promising marker for the diagnosis of AD. However, additional studies are required to confirm the sensitivity and specificity of this test.

ApoE genotyping might be reserved for patients who meet the clinical criteria for AD, as the ApoE genotype in those patients can significantly improve the specificity of the clinical diagnosis, reducing the false positive rate but also decreasing the sensitivity.

Serum melanotransferrin concentrations and CSF glycolysated AChE are elevated in AD patients. Preliminary studies of these markers seem promising, but further work is necessary.

Age-related increase in CSF S-100 concentration may be important in the pathogenesis of AD. However, increased concentrations of S-100 are also found in the CSF of patients with frontotemporal dementia or following cardiac operations or head trauma, stressing the lack of specificity of this marker.

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Accepted for publication 2 May 2000

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Arch Neurol. 1999 Mar;56(3):281-3.

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Biomarkers of Alzheimer Disease

John H. Growdon, MD

A definitive diagnosis of Alzheimer disease (AD) depends on finding widespread neurofibrillary tangles and plentiful neuritic plaques in the brain of an individual with a clinical diagnosis of progressive dementia.[1] Using contemporary diagnostic criteria, the antemortem diagnosis of probable AD in centers specialized for AD is confirmed 80% to 90% of the time. There is the suspicion, but no firm data, that diagnostic accuracy is much lower outside of practices dedicated to patients with dementia. Furthermore, the diagnostic workup is expensive. In most settings, the evaluation generally includes a careful medical history and physical examination; neurologic examination (and psychiatric consultation as indicated); laboratory blood studies to exclude underlying metabolic and medical illnesses that masquerade as AD; a mental status assessment and formal cognitive tests; and a computed tomographic scan or magnetic resonance imaging of the brain.[2] Because these procedures are time-consuming and costly, there is a need to identify biological tests that can circumvent aspects of this workup and point the physician to the correct diagnosis. It would be highly desirable to measure a subtance or substances in blood or urine samples or cerebrospinal fluid (CSF) that would lead to a positive diagnosis of AD without the need for specialized dementia clinics and the expense and time of standard diagnostic evaluations. In response to this need, the Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging convened a working group in 1997 to examine the status of various antemortem markers for AD. The consensus statement of this group, entitled "Molecular and Biochemical Markers of AD," was published in 1998.[3] The consensus statement first defined the characteristics of an ideal biomarker, and then outlined the steps required for a proposed biomarker to achieve acceptance by the medical community. Finally, the statement reviewed the current state of all proposed biological markers. The workshop participants observed that none of the current biomarkers had yet achieved universal acceptance and concluded none fully met the consensus criteria for an ideal marker. Nonetheless, several tests were identified as good markers for familial AD, and several other tests showed promise as a diagnostic aid for sporadic AD. The purpose of this review is to put these recommendations into a practical context. What does the consensus statement tell the practicing clinician? How do the opinions in the consensus statement affect clinical practice in diagnosing and treating patients with dementia?

Arch Neurol. 1999;56:281-283

The most successful diagnostic tests for AD are based on advances in molecular genetics, and are limited to early-onset familial AD. Missense mutations in 3 genes—*PS1* on chromosome 14, *PS2* on chromosome 1, and *APP* on chromosome 21—all cause familial AD. In all, only a few hundred families carry these identified mutations that cause autosomal dominant AD. Most of these pedigrees have *PS1* mutations and onset of dementia at age younger than 50 years;

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mutations in the *APP* gene are rare, and there are only 2 pedigrees with *PS2* mutations. From a practical viewpoint, it is reasonable to search for a *PS1* mutation in familial AD with very early onset, but searching for missense mutations in individuals with familial AD with onset older than 50 years or in individuals without a family history of AD is rarely worthwhile. In contrast to these deterministic genetic causes of AD, the apolipoprotein E (apoE) ε4 allele is a risk factor for AD. Numerous studies[4,5] attest to the fact that the ε4 allele is 3 to 4 times more common in AD, including the most common category of late-onset disease without known family history, than in individuals who are not demented. As opposed to the missense mutations, however, the ε4 allele is not a deterministic cause of AD, only a risk factor. Thus, relying on the *apoE* genotype alone to establish a diagnosis of AD is inadequate because this measure by itself has low sensitivity and specificity. Many individuals, perhaps a majority, who inherit ε4 do not develop AD even at an advanced age. Nonetheless, when used in conjunction with conventional diagnostic workup, finding an ε4 allele adds a small percentage of confidence to the clinical diagnosis.[6]

Genetics aside, the most convincing diagnostic tests will be those that seek to detect in life the histopathologic hallmarks characteristic of AD that are observed in the brain at death. The neurofibrillary tangle and neuritic plaque are the principal lesions associated with AD. The neurofibrillary tangle is composed primarily of hyperphosphorylated tau, which is a cytoskeletal protein. Diffuse amyloid deposits pepper the AD brain; the mature neuritic plaque contains a compacted core of amyloid protein. Much of this amyloid is a 42-amino acid peptide derived from proteolytic cleavage of a larger amyloid precursor protein molecule. This peptide, called $A\beta_{1-42}$, has putative neurotoxic properties that may initiate a cascade of events leading to neuronal dysfunction and death.[7,8] As a result of intense investigation into the mechanism whereby tangles and plaques form, antibodies directed toward epitopes of tau and toward different amyloid fragments have been adapted for clinical use. Under normal conditions, a small amount of soluble $A\beta_{1-42}$ circulates in the bloodstream. In individuals with the deterministic mutations for AD in *PS1*, *PS2*, and *APP*, $A\beta_{1-42}$ levels in the blood are increased compared with levels in sporadic AD, which generally do not differ from levels measured in individuals without dementia.[9] Although potentially useful in diagnosing familial AD, measuring $A\beta_{1-42}$ levels in the blood is limited to research laboratories and is not in widespread use. Even if this test were widely available, information to date indicates plasma $A\beta_{1-42}$ levels would not be diagnostically useful in most patients with nonfamilial or late-onset AD.

The CSF bathes the brain, and is potentially a more accurate representation of what goes on in the brain than measuring tau or Aβ in the peripheral bloodstream. Both tau and amyloid fragments can be measured in CSF; their detection forms the basis for the development of commercial diagnostic tests for AD. There is general consensus that CSF levels of tau are significantly increased in AD compared with both healthy control subjects and patients with non-AD neurologic diseases.[10] Similarly, there is general consensus that levels of $A\beta_{1-42}$ are characteristically decreased in AD, whereas levels of Aβ total ($A\beta_{1-42}$ and $A\beta_{1-40}$) are no different in patients with AD than in control subjects.[11] The crucial question is whether the increases in tau and decreases in $A\beta_{1-42}$ occur with sufficient frequency and magnitude that they offer diagnostic value. To date, the answer has been no. Both measures suffer from poor accuracy: if sensitivity is set at a satisfactory 80% to 90% level, specificity is low, and vice versa. Combining the 2 measures increase accuracy slightly, but many individual values remain in a diagnostically indeterminate range.[10,12] A further limitation on using these measures as diagnostic tests is that few cases of dementia with a clinical diagnosis of probable AD and the putative diagnostic profile of low $A\beta_{1-42}$ and high tau in CSF during life have been confirmed pathologically. Other CSF markers, such as neuronal thread protein, have been proposed as biomarkers for AD,[13] but require independent confirmation. It is likely that refinements on current $A\beta_{1-42}$, tau, or neuronal thread protein assays will occur as a result of attempts to improve diagnostic accuracy. At present, these tests can be recommended only as an adjunct to comprehensive diagnostic assessment in difficult cases. Confidence in the diagnosis increases when the CSF profile fits that expected in AD, but indeterminate results should be anticipated.

What can we expect from an ideal biomarker for AD? It is probably unrealistic to expect that any biomarker be 100% specific and sensitive for the diagnosis of AD. At autopsy, the brains of many patients with definite AD have other lesions, including infarcts, gliosis, and Lewy bodies. In any given instance, it is difficult to be certain which of these was etiologically important in producing dementia. Clinicians may eventually follow the lead of pathologists, who have adopted a less dichotomous diagnosis of AD vs no AD than in the past. According to the National Institute on Aging and the Reagan Research Institute of the Alzheimer's Association consensus criteria for the neuropathological diagnosis of AD,[14] the diagnosis of AD is probabilistic. That is to say, the diagnosis of AD is based on a likelihood estimate in which all pathologic findings are described, and the extent and intensity of plaques and tangles graded. If pathologists examining the entire brain are cautious in diagnosing AD unequivocally, then perhaps clinicians, armed with indirect molecular and biochemical measures of plaques and tangles, should be even more reserved. Viewed from this perspective, biological markers of AD may turn out to be more useful in tracking the course of illness and documenting the response to treatment than in diagnosis.

Accepted for publication June 20, 1998.

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EMBO J., 14:918-26, 1995

J Cell Biol., 143:49-62, 1998

J. Biol Chem 275:30451-57, 2000

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ACKNOWLEDGEMENTS. A.A.H. and K.M. contributed equally to this work. We are grateful to H. Lechner for introduction to the CBF3 preparation and for helpful discussion. We thank P. Sorger for help with molecular biology, T. Shimizu for help with ATP analogue experiments, and L. Cramer, R. Deshaies, A. Murray, K. Sawin, T. Stearns, V. Gelfand and P. Peluso for critical reading of the manuscript. A.A.H. is a Lucille P. Markey Scholar; J.C. is an American Cancer Society professor; and J.C. and T.J.M. are funded by grants from the NIH and the Packard and Searle foundations.

CENP-E is a putative kinetochore motor that accumulates just before mitosis

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THE mechanics of chromosome movement, mitotic spindle assembly and spindle elongation have long been central questions of cell biology[1]. After attachment in prometaphase of a microtubule from one pole, duplicated chromosome pairs travel towards the pole in a rapid but discontinuous motion[2,3]. This is followed by a slower congression towards the midplate as the chromosome pair orients with each kinetochore attached to the microtubules from the nearest pole. The pairs disjoin at anaphase and translocate to opposite poles and the interpolar distance increases. Here we identify CENP-E as a kinesin-like motor protein (M_r 312,000) that accumulates in the G2 phase of the cell cycle. CENP-E associates with kinetochores during congression, relocates to the spindle midzone at anaphase, and is quantitatively discarded at the end of the cell division. CENP-E is likely to be one of the motors responsible for mammalian chromosome movement and/or spindle elongation.

To identify novel proteins of the human centromere-kinetochore complex, we generated monoclonal antibodies against fractionated chromosomal proteins and used immunofluorescence to isolate antibodies that identify antigens localized at the centromeres. One such monoclonal antibody (177) identified CENP-E, a protein of M_r 312K that could not be detected in interphase cells, but first appeared at the centromere regions of chromosomes in prometaphase[4]. To determine the primary structure of CENP-E, antibody 177 was used to screen a human complementary DNA expression library. A 2-kilobase (kb) cDNA (clone P2, Fig. 1*a*) identified an ~8 kb HeLa cell RNA (data not shown), large enough to encode CENP-E. To verify whether this partial cDNA encoded a portion of CENP-E, a rabbit polyclonal antiserum (pAb1) was raised against a bacterially produced protein encoded by a subclone of the P2 cDNA (stippled domain in Fig. 1*a*) that did not encode the antibody 177 epitope. On immunoblots of chromosomal proteins this antiserum identified a high-molecular-mass protein, whose size was indistinguishable from the antigen identified using antibody 177 (Fig. 2*a*), and which stained the centromere region of isolated chromosomes (Fig. 2*b*). The polyclonal antiserum also revealed (Fig. 2*c*) the same cell cycle-dependent localization of the antigen in HeLa cells as was seen previously with antibody 177 (ref. 4). The recognized antigen was localized to a set of punctate dots on prometaphase and metaphase chromosomes. At anaphase, the antigen became detached from chromosomes and localized exclusively to fibres in the spindle midzone and to the midbody during telophase. We conclude that this cDNA clone is derived from a portion of the CENP-E messenger RNA.

By rescreening the original cDNA library, and by construction of a sub-library primed by a CENP-E-specific oligonucleotide, a set of eight overlapping cDNAs which spanned a total of 8.3 kb were obtained (Fig. 1*a*). DNA sequencing revealed a composite open reading frame (encoding 2,663 amino acids)

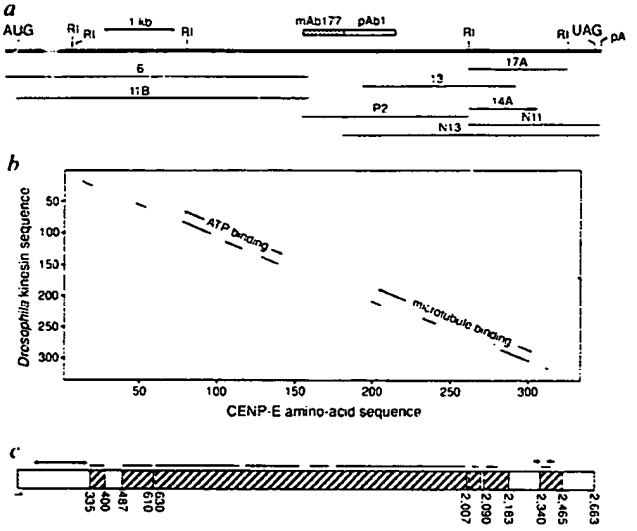


FIG. 1 Schematic diagram of CENP-E cDNA clones, comparison of the CENP-E and kinesin heavy-chain motor domains, and predicted structure of the CENP-E polypeptide. *a*, Schematic drawing of eight overlapping cDNA clones that span the 8,371-base CENP-E messenger RNA. Positions of translation initiation (AUG) and termination (UAG) and the putative polyadenylation signal (pA) are noted. RI, positions of *Eco*RI sites. Stippled box denotes the domain used to generate pAb1, a rabbit polyclonal antibody against bacterially expressed CENP-E. The cross-hatched domain denotes the region that contain the antibody 177 epitope. *b*, Dot matrix homology plot (Macvector, |B|) comparison of the motor domains (335 amino acids) of *Drosophila* kinesin heavy-chain and CENP-E. The portions of *Drosophila* kinesin that represent its known ATP and microtubule binding domains[10] are noted. *c*, Predicted secondary structure of the CENP-E polypeptide. Cross-hatched domains are predicted to adopt α-helical structures[5]. Overlined domains are predicted to adopt coiled-coil conformations[6]. Arrows above denote regions of >50% sequence identity to *Drosophila* kinesin heavy chain.

METHODS. Plaques (5×10^5) from a λgt11 human breast carcinoma cDNA library (Clontech) were probed with antibody 177 after induction of fusion protein expression with isopropyl-β-D-thiogalactoside. Immunopositive clones were identified with ^{125}I-labelled goat anti-mouse antibodies (Amersham). After plaque purification, the cDNA from the initial cDNA clone (P2) was used to rescreen the same library. To extend the P2 cDNA towards the 5′ end of the mRNA, an oligonucleotide corresponding to the sequence near the 5′ end of P2 was used as a primer for reverse transcription of poly(A)$^+$ RNA obtained from HeLa cells synchronized in G2. Synthesis of cDNA was done using a kit (Pharmacia) and after addition of *Not*I/*Eco*RI adaptors, the cDNA was ligated into *Eco*RI-digested ZAPII and packaged into phage particles (Stratagene). Unamplified plaques (10^5) were screened using a 5′ portion of the P2 cDNA. The authenticity of each new clone was initially verified by its ability to identify the same 8–9 kb RNA on RNA blot analysis. Subsequently antibodies raised against bacterially derived fusion proteins encoded by each new cDNA portion were shown to recognize CENP-E.

preceded by in-frame translation terminators (Fig. 3). The predicted CENP-E polypeptide structure[5] consists of three domains: amino- and carboxy-terminal globular domains, each of ~500 amino-acid residues, separated by a central 1,700 amino-acid segment which may form a discontinuous α-helix (Fig. 1*c*). This helical domain contains an extensive, hydrophobic heptad repeat characteristic of coiled-coil helices[6]. Comparison of the sequence of CENP-E with all sequences in current gene and protein data bases revealed that the N-terminal 335 amino acids share extensive homology with the motor domain of all known kinesin-like proteins[7-16]. Within this motor region, CENP-E shares virtual sequence identity (Fig. 1*b*) with the 120 residues that comprise the highly conserved nucleotide- and microtubule-binding sites characteristic of the kinesin family of proteins[17]. In sequence and in predicted structure, CENP-E is most like the conventional kinesin heavy-chain, although its α-helical 'rod' segment is nearly four times longer than that of kinesin. Unlike other kinesin-like proteins, sequence comparison revealed an additional 18-amino-acid segment near the C terminus of CENP-E (Fig. 1*c*; residues 2,343–2,360) with 50% sequence identity (78% if highly conservative substitutions are counted) to sequences in the C termini of heavy-chain kinesins[10,11,15,16]. As kinesin light-chains bind in this globular tail region[18], this conserved domain may represent a binding site for kinesin light-chains or a related accessory factor(s).

That CENP-E seems to be absent from ~90% of interphase cells (for example, arrowed cells in bottom panels of Fig. 2*c*; see also ref. 4) suggests that the abundance of CENP-E might be dependent on the phase of the cell cycle. To test this directly, HeLa cells were synchronized at the G1/S boundary and after release, extracts from equal numbers of cells at various points in the cycle were examined for accumulated CENP-E. Cells in the G1 and early S phases showed little detectable CENP-E in either cytoplasmic or nuclear fractions, but cytoplasmic CENP-E levels rose sharply during late S and G2/M (Fig. 4*a*, upper panel), ultimately reaching the level found in cells arrested in prometaphase by inhibition of spindle assembly. At all times CENP-E is excluded from nuclei (Fig. 4*a*, lower panel), consistent with the absence of a consensus nuclear targeting signal[19-21].

To examine the loss of CENP-E at the end of mitosis, CENP-E synthesized during late S phase was pulse-labelled and followed during the subsequent cell cycle. After 1 h, when most cells were still in G2 or had just entered mitosis, CENP-E synthesized earlier remained stable (Fig. 4*b*). When most cells had completed mitosis, only ~20% of CENP-E synthesized in the preceding G2 phase remained. This loss of CENP-E was dependent on completion of mitosis, as CENP-E remained stable in cells blocked in mitosis with colcemid. Even after a 4 h chase, when virtually all CENP-E was lost from cells that had completed mitosis, CENP-E remained in colcemid-arrested cells (Fig. 4*b*).

The ability of CENP-E to interact with microtubules in HeLa cell extracts was tested. CENP-E quantitatively sedimented after taxol-stimulated microtubule assembly (Fig. 4*c*, lane 2), but not when microtubule assembly was blocked by the addition of colcemid (Fig. 4*c*, lane 3). As with other kinesin-like proteins, CENP-E still co-sedimented with microtubules after the addition of 1 mM β-γ-imidoadenosine 5'-phosphate (AMP-PNP), an inactive ATP analogue, or depletion of ATP (Fig. 4*d*, lanes 6, 9). But addition of 10 mM ATP released only ~50% of microtubule-bound CENP-E (Fig. 4*d*; compare lanes 10 and

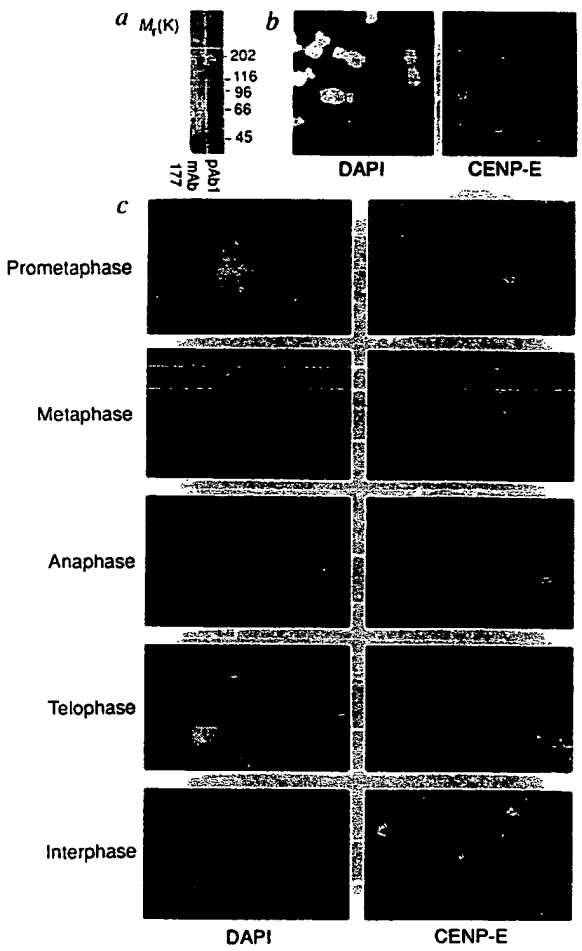
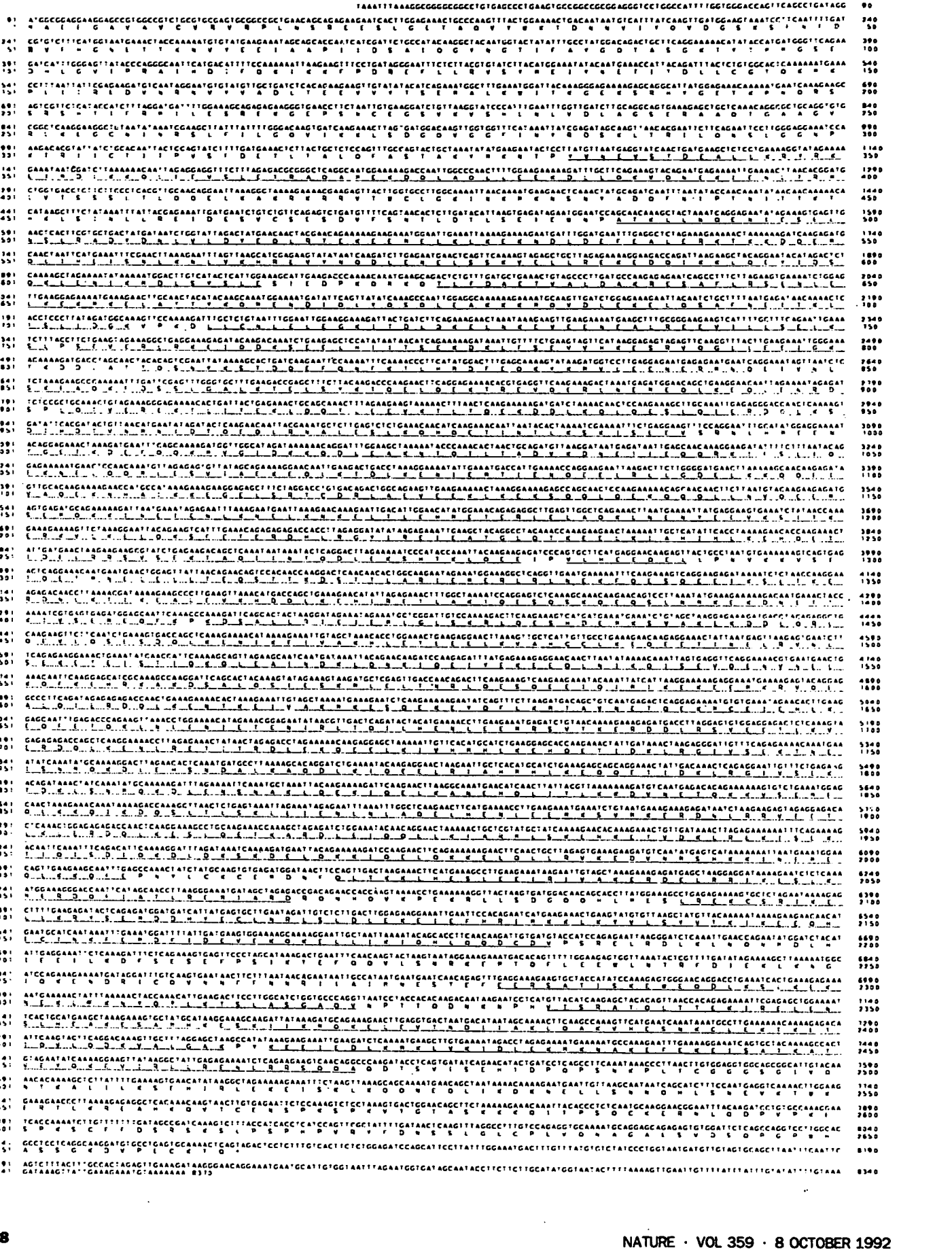


FIG. 2 Authentication of CENP-E cDNA clones. *a*, Immunoblotting of HeLa chromosomal proteins using (lane 1) pAb1 and (lane 2) antibody 177, a polyclonal antibody generated against a portion (stippled domain in Fig. 1*a*) of the P2 cDNA that does not encode the antibody 177 epitope. Migration position of myosin (M_r 202K) is shown to the left. *b* (left), DAPI stain of chromosomes and (right) indirect immunofluorescence localization of CENP-E at the centromeres of isolated HeLa chromosomes using pAb1. Scale bar, 5 μm. *c*, Indirect immunofluorescence localization on CENP-E in HeLa cells at various points in the cell cycle and visualized using pAb1. Scale bar, 10 μm. Arrows in lower panels mark interphase cells that do not stain for CENP-E.

METHODS. Chromosomal proteins were prepared from HeLa cells, immunoblotted[4] and then incubated either with antibody 177 or pAb1, a polyclonal antibody generated against a fusion protein made up of the bacterial protein trpE linked in frame with the polypeptide encoded by the 863-bp *Xho*I–*Hin*dIII fragment of cDNA clone P2 (stippled region in Fig. 1*a*). Primary antibody was detected with ^{125}I-labelled secondary antibodies or protein A. To produce pAb1, the P2-encoded protein, expressed using the pATH plasmid vectors and the protease-deficient *Escherichia coli* strain CAG456, was gel-purified and rabbits were immunized. Indirect immunofluorescence staining of isolated HeLa chromosomes and cells was done as described[4], except that rabbit polyclonal antibodies (*a*) were used as the primary antibody, followed by biotinylated anti-rabbit antibodies (Vector) and streptavidin conjugated with Texas Red (Sigma). Slides were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics and images photographed using Kodak Tmax 400 film.



◀ FIG. 3 Nucleotide and deduced amino-acid sequence of CENP-E. Composite nucleotide and predicted amino-acid sequence of CENP-E were determined by sequencing the overlapping cDNAs shown in Fig. 1a. Underlined sequences are predicted[6] to form an α-helical, coiled-coil conformation. All regions were sequenced on both strands using the chain-termination method.

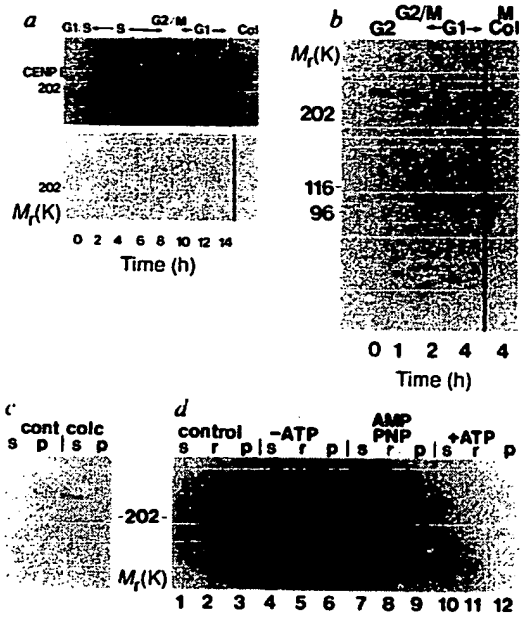


FIG. 4 Cell cycle-dependent accumulation and loss, and microtubule binding of CENP-E. *a*, Steady-state levels of CENP-E in the cytoplasm (upper panel) and nuclei (lower panel) of cells at different stages of the cell cycle, marked above. Col, cells blocked in mitosis by addition of colcemid. *b*, The turnover of CENP-E at the end of mitosis observed by pulse-chase and immunoprecipitation. *c*, Cosedimentation of CENP-E with microtubules assembled in HeLa extracts of control (cont) and colcemid-treated (colc) cells. *d*, Nucleotide sensitivity of the interaction between CENP-E and microtubules; s, supernatant; r, rinse; p, pellet.

METHODS. *a*, HeLa cells grown in suspension were incubated in excess thymidine (2.5 mM). The cells were released into complete media for 9 h and aphidocolin (Calbiochem) was added to a final concentration of 5 μg ml^{-1} to synchronize cells at the G1/S boundary. Cells were collected every 2 h after release from the block and cell synchrony was monitored by flow cytometry. To measure the steady-state levels of CENP-E, synchronized cells were lysed in 2 ml of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.5% deoxycholate, 1.0% NP-40, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg ml^{-1} each of leupeptin, aproptinin and pepstatin) and centrifuged at 15,000*g* for 20 min. The pelleted nuclei and insoluble debris were boiled in SDS-sample buffer, separated by electrophoresis and transferred onto Immobilin P (Millipore) for immunoblot analysis. Cytoplasmic extracts were incubated with pAb1 (1:200 final) at 4 °C for 2 h. A 1 : 1 slurry (40 μl) of washed Protein A Sepharose (Pharmacia) beads were added and the immune complex was washed in RIPA buffer, boiled in SDS-sample buffer, separated on a 4–10% gradient gel, transferred onto the membrane and processed for immunoblot analysis using the same antibody that was used for the immunoprecipitation (used at a 1:1,000 dilution). ^{125}I-labelled protein A (ICN) was used to detect bound primary antibodies. For pulse-chase experiments, cells in G2 were radiolabelled for 1 h using Trans-label (ICN) at a final concentration of 100 μCi ml^{-1}. After labelling, an aliquot of cells was immediately lysed. The remainder were released into complete media until the end of mitosis. To block cells in mitosis, cells were chased in unlabelled media that contained 0.5 μg ml^{-1} of colcemid (Gibco). Cell lysates were processed for immunoprecipitation as described. For analysis of the sedimentation of CENP-E with microtubules, 1×10^9 HeLa cells were pelleted, washed, and the packed cell pellet was resuspended in an equal volume of homogenization buffer (80 mM PIPES, pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA, 0.1 mM dithiothreitol, 1 mM PMSF and 1 μg ml^{-1} each of leupeptin, aprotinin and pepstatin). An S-100 extract was incubated with taxol (10 μM final) to induce microtubule polymerization. Microtubules were pelleted by centrifugation at 100,000*g*. Supernatants were saved and the pellets were rehomogenized in taxol-containing buffer and recentrifuged as described. To detect CENP-E, the supernatants were combined and adjusted to 1 × RIPA buffer. The pellets were rinsed with 1 ml of polymerization buffer and directly solubilized with RIPA buffer. CENP-E was immunoprecipitated and processed for immunoblot analysis as described above. For ATP depletion, 20 U ml^{-1} hexokinase and 20 mg ml^{-1} glucose were added to the S-100 extracts for 15 min before inducing microtubule polymerization. In some experiments, AMP-PNP or Mg-ATP was present during the polymerization reaction at final concentration of 1 mM or 10 mM, respectively. To block microtubule assembly, colcemid (final concentration 1 μg ml^{-1}) was added to the S-100 extracts.

12), similar to the situation found previously for the *non-claret disjunctional* (*ncd*) protein[12]. The nucleotide-dependent partial release of CENP-E indicates that CENP-E might interact with microtubules directly or indirectly through a nucleotide-insensitive site. This is supported by the fact that the C-terminal 95 amino acids that comprise a basic (pI 9.3), proline-rich (13.4%) domain, similar to the putative microtubule binding sites of tau and MAP2 (ref. 22).

What is the role of CENP-E during mitosis? The striking cell cycle-dependent accumulation and loss of CENP-E, and the presence of CENP-E at kinetochores during chromosome congression and at the midzone during spindle elongation, give strong support to the view that CENP-E is an important motor molecule in chromosome movement and/or spindle elongation. Consistent with this, microinjection of one CENP-E antibody transiently arrests cells at metaphase[4]. Further experiments to demonstrate motor activity, to determine the direction of CENP-E mediated movement, and to inhibit CENP-E function *in vivo* are now needed to document more clearly the role(s) of CENP-E. □

Received 22 April; accepted 27 August 1992.

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ACKNOWLEDGEMENTS. We thank L. Wordeman for advice in the microtubule binding assay. Taxol was a gift from the National Cancer Institute. This work has been supported by grants from the L. P. Markey Foundation to T.J.Y. and NIH to D.W.C. B.T.S. was supported by a pre-doctoral NIH training grant.

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Nature 359, 536-39, 1992

EMBO J., 14:918-26, 1995

J Cell Biol., 143:49-62, 1998

J. Biol Chem 275:30451-57, 2000

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The EMBO Journal vol.14 no.5 pp.918–926, 1995

Mitotic HeLa cells contain a CENP-E-associated minus end-directed microtubule motor

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Communicated by M.Osborn

A minus end-directed microtubule motor activity from extracts of HeLa cells blocked at prometaphase/metaphase of mitosis with vinblastine has been partially purified and characterized. The motor activity was eliminated by immunodepletion of Centromere binding protein E (CENP-E). The CENP-E-associated motor activity, which was not detectable in interphase cells, moved microtubules at mean rates of 0.46 μm/s at 37°C and 0.24 μm/s at 25°C. The motor activity co-purified with CENP-E through several purification procedures. Motor activity was clearly not due to dynein or to kinesin. The microtubule gliding rates of the CENP-E-associated motor were different from those of dynein and kinesin. In addition, the pattern of nucleotide substrate utilization by the CENP-E-associated motor and the sensitivity to inhibitors were different from those of dynein and kinesin. The CENP-E-associated motor had an apparent native molecular weight of 874 000 Da and estimated dimensions of 2 nm × 80 nm. This is the first demonstration of motor activity associated with CENP-E, strongly supporting the hypothesis that CENP-E may act as a minus end-directed microtubule motor during mitosis.

Key words: CENP-E/dynein/kinesin/microtubule motor/mitosis

Introduction

Centromere binding protein E (CENP-E), initially identified using a monoclonal antibody (177) raised against fractionated chromosomal proteins (Yen *et al.*,1991), is a 312 kDa protein that belongs to the kinesin superfamily (Yen *et al.*, 1992). The N-terminal region of CENP-E shares strong sequence homology with the microtubule motor domain of kinesin and kinesin-like proteins (KLPs) (Goldstein, 1993) and binds to microtubules in an ATP-sensitive manner (Liao *et al.*, 1994). Thus it has been suggested that CENP-E, like kinesin, may be a microtubule motor.

CENP-E is present in the cytoplasm of HeLa cells at very low levels during G_1 or early S phase and its expression increases during late S phase and G_2, reaching a peak at the transition between G_2 and mitosis (Brown *et al.*, 1994). By immunofluorescence microscopy with a monoclonal antibody specific for CENP-E (antibody 177), CENP-E was found to localize initially at the kinetochores of chromosomes during early prometaphase of mitosis. It remains at kinetochores until anaphase, when it becomes redistributed to the spindle midzone. It then becomes localized in the midbody at the end of mitosis (Yen *et al.*, 1991) and appears to be degraded following anaphase (Brown *et al.*, 1994). CENP-E appears to play a role in chromosome movement during mitosis (Yen *et al.*, 1992). For example, injection of antibody 177 into cells during prometaphase delays or prevents progression from metaphase to anaphase (Yen *et al.*, 1991).

In the present work, we describe the properties of a motor activity that is enriched in extracts from HeLa cells blocked in prometaphase/metaphase with 2–5 nM vinblastine. The motor moves along microtubules towards the minus ends of microtubules and the motor activity is depleted from HeLa extracts by an antibody specific for CENP-E. CENP-E co-purifies with the motor activity during a microtubule-affinity purification step and by sucrose density gradient fractionation, suggesting that the motor activity may be due to CENP-E.

Results

Extracts of mitotic HeLa cells contain a microtubule motor activity that is not detectable in interphase cell extracts

We unexpectedly detected microtubule motor activity in supernatants of centrifuged lysates of mitotic HeLa cells (S-100 extracts, Materials and methods) while analyzing the effects of the extracts on dynamic instability behavior of microtubules *in vitro*. The activity was barely detectable in the extracts because a significant proportion of the microtubules was immobilized by association with large membrane-like structures similar to those observed in *Xenopus* egg extracts (Allan and Vale, 1991). Fractionation of the mitotic S-100 extracts by DEAE–cellulose column chromatography yielded a fraction (D-100, Materials and methods) which did not contain microtubule-associated membranous networks. When taxol-stabilized bovine brain microtubules were added to the D-100 mitotic fractions on coverslips in the presence of 1 mM ATP and examined by video microscopy, >90% of the microtubules exhibited gliding behavior. The microtubules glided continuously in a directional manner indicating that the gliding was caused by a microtubule motor.

The motor activity in D-100 fractions from mitotic extracts moved microtubules in the presence of 1 mM ATP at mean rates of 0.46 ± 0.03 μm/s at 37°C (Figure 1) and 0.24 ± 0.02 μm/s at 25°C (data not shown). The rate

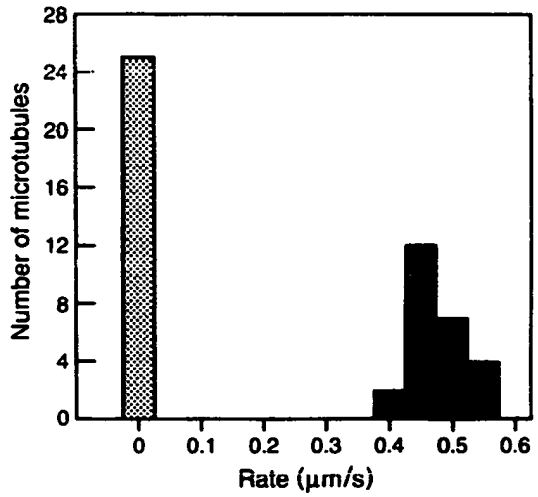

Fig. 1. Microtubule gliding supported by mitotic but not interphase HeLa cell extracts. HeLa mitotic D-100 fractions (solid bars) (8 mg/ml total protein) or D-100 fractions of interphase cells (stippled bar) (8 mg/ml total protein) were incubated on coverslips for 15 s. Solutions of taxol-polymerized bovine brain microtubules in 1 mM ATP were then added. The mean (standard deviation) rate of movement of 25 microtubules in mitotic extracts at 37°C was 0.46 ± 0.03 μm/s. No movement could be detected with 25 microtubules in D-100 interphase extracts.

of microtubule movement did not increase when the D-100 fractions were concentrated 4-fold or when the ATP concentration was increased to 10 mM. The motor activity is ~4-fold slower than that of HeLa cell dynein (described below) and is slower than that of kinesin (0.4–0.9 μm/s at 22–25°C, Vale *et al.*, 1985; Porter *et al.*, 1987; Saxton *et al.*, 1988). D-100 fractions of interphase cells prepared in a manner identical to D-100 fractions of mitotic cells had no detectable microtubule motor activity (Figure 1).

The D-100 mitotic motor is minus end-directed

The direction of the HeLa mitotic motor-induced movement was determined with an axoneme-based gliding assay (Pryer *et al.*, 1986). Growth of bovine brain tubulin onto the plus and minus ends of the axonemes was carried out (without taxol) at a tubulin concentration of 10 μM, which resulted in the formation of microtubules which were longer at the plus ends of axonemes and much shorter at the minus ends of axonemes. When D-100 fractions of HeLa mitotic cells were added to coverslips followed by addition of the axoneme constructs, the axonemes glided with their plus ends leading, i.e. minus end-directed movement (Figure 2). Plus end-directed motion was never observed.

To eliminate the possibility that the minus end-directed axoneme movement was due to axoneme-associated dynein, gliding assays were performed with the coverslips coated with bovine serum albumin instead of the D-100 fractions. No axonemes moved under these conditions (data not shown). To verify the direction of movement we constructed axonemes with microtubules at plus ends only using bovine brain tubulin treated with *N*-ethylmaleimide (NEM) (Hyman *et al.*, 1991) and again found that the axonemes moved with microtubule plus-ends leading (data not shown).

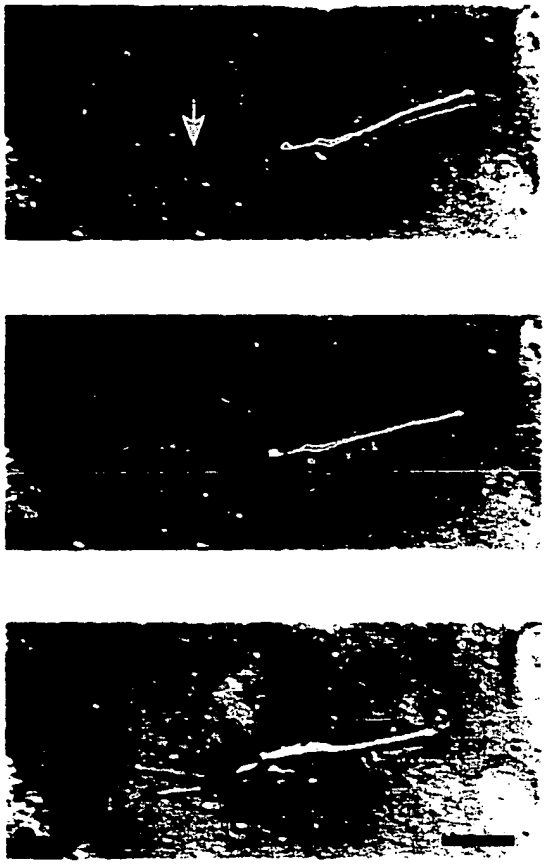

Fig. 2. Microtubule motility in mitotic HeLa cell extracts is minus-end directed. 1 μl volumes of a D-100 fraction were applied to coverslips and allowed to adsorb to the surface for 15–30 s. Microtubule–axoneme constructs prepared without taxol were then diluted into PEM_{50} buffer containing 20 μM tubulin, 2 mM GTP and 2 mM ATP, and 1 μl volumes of this mixture were added to the D-100 extract on the coverslips. (a)–(c) are video microscopy images taken at 5 s intervals at 25°C showing the movement of one such axoneme. The two ends of the axoneme are marked with dark arrows and a bovine brain microtubule at the axoneme plus end is marked with a white arrow. The dark arrowhead marks an object on the surface of the coverslip that did not move and serves as a reference point for movement of the axoneme. The axoneme moved with its plus end (long microtubules) leading. Movement of a total of 45 axoneme constructs with three different preparations of the D-100 fraction were evaluated. Only minus-end directed movement occurred. Bar = 3 μm.

The D-100 mitotic motor is associated with CENP-E

CENP-E has been postulated to be a microtubule motor based upon sequence similarity to the motor domain of kinesin (Yen *et al.*, 1991). Thus, we examined whether the motor activity present in the HeLa mitotic extracts might involve CENP-E by immunodepleting any CENP-E from the extracts. We used a polyclonal antiserum, HX-1, specific for the putative stalk domain of CENP-E (see Materials and methods). As shown in Figure 3, lanes 1 and 2, HX-1 quantitatively depleted CENP-E protein from the mitotic D-100 fractions. When the CENP-E-depleted D-100 fractions were examined for the ability to effect microtubule movement, no movement was detected (Figure 3, microtubule gliding motility is indicated for each supernatant fraction below the lane number; motility was quantitated as the reciprocal of the greatest dilution

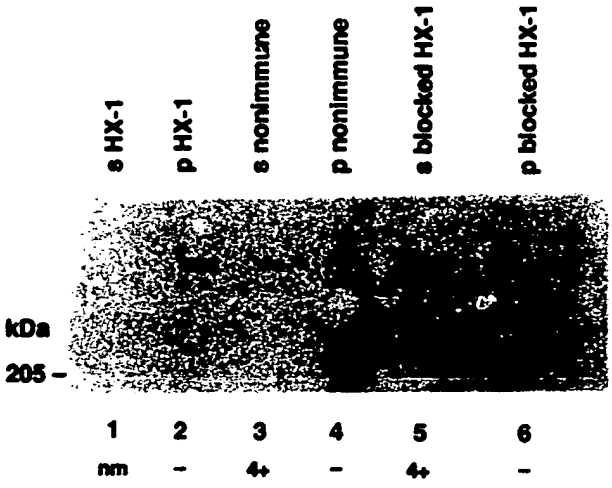

Fig. 3. Immunoprecipitation of CENP-E from D-100 fractions. D-100 fractions were immunoprecipitated by adding HX-1 antiserum and *Staphylococcus* protein A–Sepharose to the fractions followed by centrifugation (Materials and methods). The resulting pellets and supernatants were subjected to SDS–PAGE and immunoblotting with HX-1 antiserum. Microtubule gliding was assayed in the supernatants and the motility is shown for each lane below the lane number. Fractions with 4+ motility were active at a 4-fold dilution (Porter *et al.*, 1987), as was the D-100 extract prior to immunoprecipitation (not shown). nm, no motility. Fractions designated (–) were not assayed for motility. s HX-1 (lane 1), supernatant from the HX-1 immunoprecipitation; p HX-1 (lane 2), pellet from the HX-1 immunoprecipitation; s non-immune (lane 3), supernatant from the non-immune serum immunoprecipitation; p non-immune (lane 4), pellet from the non-immune serum immunoprecipitation; s blocked HX-1 (lane 5) supernatant from the HX-1 fusion protein-blocked HX-1 antibody; p blocked HX-1 (lane 6) pellet from the HX-1 fusion protein-blocked HX-1 antibody.

which supported motility). Non-immune control serum did not precipitate CENP-E protein from the mitotic D-100 fractions (Figure 3, lanes 3, 4), and did not remove any microtubule gliding activity. In addition, incubation of the HX-1 antiserum with the HX-1 fusion protein prior to adding the antiserum to the D-100 fractions prevented depletion of CENP-E (Figure 3, lane 5) and had no effect on the microtubule movement. These data indicate that the motor present in the HeLa mitotic D-100 fractions is associated with CENP-E.

Characterization of the CENP-E-associated motor

Nucleotide specificity. Previous studies have shown that different molecular motors have distinct pharmacological 'fingerprints' (Shimizu *et al.*, 1991). We characterized the motility of the CENP-E-associated motor in D-100 mitotic extracts with various nucleotide substrates to determine how the motor activity compared with the activities of kinesin and dynein. We found that the nucleotide specificity of the CENP-E-associated motor was different from that of either dynein or kinesin (Table I). For example, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), which facilitates movement with dynein, did not promote movement with the CENP-E-associated motor (Table I). In contrast to kinesin (Shimizu *et al.*, 1991), neither GTP, 8-bromo ATP nor 1,N^6-etheno-adenosine 5′-triphosphate (etheno ATP) promoted movement with the CENP-E-associated motor (Table I).

Inhibitor specificity. Known inhibitors of kinesin and dynein motor activities were also used to examine the similarities and differences between the CENP-E-associated motor and kinesin and dynein. The pattern of inhibition of the CENP-E-associated motor activity was different from that previously reported for kinesin or dynein. In contrast to kinesin, yet similar to dynein (Vale and Toyoshima, 1989), the activity of the CENP-E-associated motor was completely inhibited by 10 μM sodium orthovanadate (Table II). In contrast to dynein but similar to kinesin, the activity of the CENP-E-associated motor was completely inhibited by 1 mM 5′-adenylylimidodiphosphate (AMP-PNP), and was not inhibited by 1 mM NEM or 1 mM erythro-9-[3-(2-hydroxynonyl)] adenine (EHNA) (Table II). We also found that 1 mM AMP-PNP induced a rigor state with the CENP-E-associated motor that was similar to that produced with kinesin (Porter *et al.*, 1987) in which microtubules became bound to the surface of the coverslips. In contrast, high concentrations of AMP-PNP promote release of the microtubules from dynein-coated coverslips (Mitchell and Warner, 1981). Thus the pattern of inhibition of the CENP-E-associated motor activity is distinct from that of dynein or kinesin.

Conditions that inactivate or block dynein motility do not affect the CENP-E-associated motility in mitotic D-100 fractions

Based upon the foregoing results, the mitotic motor activity present in the HeLa mitotic extracts did not appear to be due to dynein. However, because the motor activity was minus-end directed and because dynein-like motors have been detected in extracts of cultured chick embryo fibroblasts and sea urchin eggs (Schroer *et al.*, 1989; Gliksman and Salmon, 1993), we used two different strategies to eliminate the possibility that a form of dynein was responsible for the gliding activity in the HeLa mitotic extracts.

Dynein is inactivated by vanadate–UV-photocleavage of the dynein heavy chain (Gibbons *et al.*,1987). Thus, in the first strategy, we determined whether photo-inactivation of any possible dynein in the D-100 fraction could destroy or alter gliding motility. Under the conditions used, exposure of purified HeLa dynein to UV irradiation in the presence of sodium orthovanadate cleaved the dynein into two fragments as expected (data not shown). Dynein that was not photo-inactivated supported microtubule gliding (Figure 4B) whereas no microtubule gliding was observed with HeLa dynein that had been UV irradiated in the presence of orthovanadate (Figure 4A). In contrast, microtubule motor activity in D-100 extracts was unchanged following incubation with orthovanadate and UV irradiation (Figure 4C and E).

Antibodies to dynein are available that are capable of blocking dynein-mediated microtubule motility (Vaisberg *et al.*, 1993). Thus in a second strategy, we used an antibody to the phosphate binding loop of *Dictyostelium* dynein (Bob), to determine whether the microtubule gliding activity in the D-100 fractions could be blocked by the antibody (Figure 4). Addition of the anti-dynein antibody Bob to the D-100 fraction had no effect on microtubule gliding (Figure 4, compare E with C). In contrast, the Bob antibody completely blocked dynein-mediated microtubule gliding (Figure 4, compare F with

Table I. Nucleotide dependence of the CENP-E-associated motor

Nucleotide	CENP-E-associated motor[a]		Kinesin[b]		Dynein[b]	
	Rate[c]	%[d]	Rate[c]	%[d]	Rate	%[d]
ATP	0.24 ± 0.02	100	0.42 ± 0.06	100	4.5 ± 1.0	100
GTP	0	0	0.11 ± 0.06	27	0	0
2'dATP	0.21 ± 0.02	88	0.38 ± 0.07	91	1.3 ± 0.3	28
3'dATP	0.20 ± 0.02	83	0.30 ± 0.06	72	2.0 ± 0.5	44
ddATP	0.15 ± 0.01	62	0.29 ± 0.05	69	0.9 ± 0.3	21
8-Bromo ATP	0	0	0.01 ± 0.01	3	0	0
8-Azido ATP	0	0	0	0	0	0
Etheno ATP	0	0	0.05 ±0.03	13	0	0
ATPγS	0	0	0.01 ± 0.01	2.2	0.21 ± 0.1	4.6

[a]CENP-E-associated motor gliding rates were measured at 25°C as described in Materials and methods. Rates are mean and standard deviation.
[b]Kinesin and *Tetrahymena* 22S ciliary dynein rates (μm/s) determined by Shimizu *et al.* (1991). Kinesin rates were measured at 22–24°C and dynein rates at 25°C.
[c]Rates are the mean and standard deviation in μm/s from three different mitotic D-100 fractions; a total of 45 microtubules were analyzed for each condition.
[d]Percent of the rate of movement induced by 1 mM ATP.

Table II. Effects of microtubule motor inhibitors on the rate of CENP-E-associated motor-induced movements

Inhibitor	Rates of microtubule gliding (as % untreated control)		
	CENP-E-associated motor[a]	Kinesin[b]	Dynein[b]
AMP-PNP (1 mM)	0	0	100
Vanadate (10 μM)	0	100	0
NEM (1 mM)	98	94	0
EHNA (1 mM)	96	104	35

[a]CENP-E motor rates were obtained as described in Materials and methods. Gliding motility was assayed in three different D-100 mitotic extracts; a total of 45 microtubules were analyzed for each condition.
[b]Data of Vale and Toyoshima (1989).

B). A non-immune serum control did not block dynein-mediated motor activity (data not shown). Thus, we can conclude that the CENP-E-associated motility in the HeLa mitotic D-100 fractions was not due to dynein.

Further purification and characterization of CENP-E-associated motor activity

Microtubule-affinity purification. CENP-E has been shown to bind to microtubules in an ATP-sensitive manner (Yen *et al.*, 1992). Thus, the affinity of CENP-E for bovine brain microtubules and its release with 10 mM Mg^{2+}ATP and 0.5 M NaCl was used to further purify the CENP-E-associated motor from mitotic D-100 fractions (Materials and methods). D-100 mitotic fractions were incubated with microtubules and the microtubules with bound motor activity were sedimented through sucrose cushions and washed in PEM_{100} buffer to remove non-microtubule-bound proteins. The CENP-E bound quantitatively to the microtubules and the CENP-E was mostly depleted from the D-100 supernatant after sedimentation of the microtubules (data not shown). Motor activity could not be eluted with PEM_{100} buffer but was readily eluted from the microtubules with PEM buffer containing 10 mM Mg^{2+}ATP and 0.5 M NaCl. Approximately 70% of the CENP-E that was originally bound to microtubules was

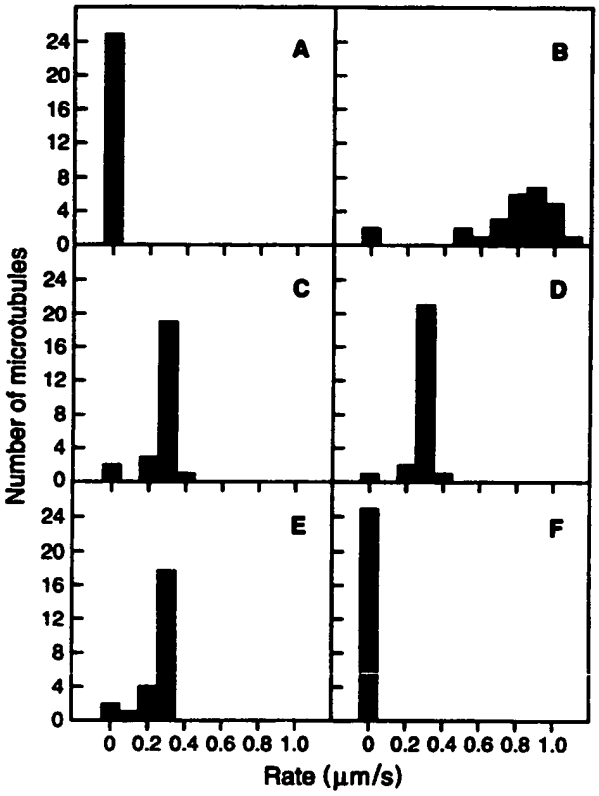

Fig. 4. Comparison of microtubule gliding induced by CENP-E containing D-100 fractions or by purified HeLa cell dynein before and after inactivation of dynein. Dynein-based motility was inactivated by UV irradiation in the presence of sodium orthovanadate (A) or by incubation with a dynein motility-blocking antibody, Bob (F). Untreated dynein (B), UV–vanadate-treated dynein (A), Untreated D-100 fraction (C), UV–vanadate treated D-100 fraction (D). D-100 fraction (E) and dynein (F) incubated with a dynein motility-blocking antibody. UV–vanadate treatment inhibited motility of dynein (compare B with A) but did not inhibit motility of D-100 fractions (compare D with C). The dynein antibody Bob blocked dynein induced motility (compare F with B) but not that of D-100 extracts (compare E with C).

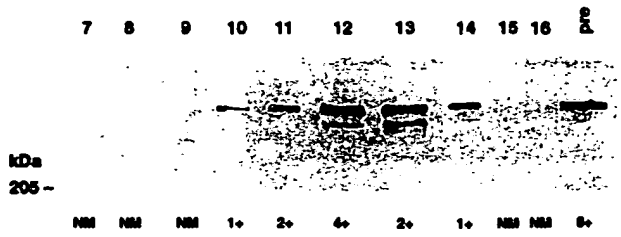

Fig. 5. Sucrose density centrifugation of microtubule affinity-purified CENP-E-associated motor activity. CENP-E-associated motor activity was eluted from microtubules with a solution of 10 mM Mg^{2+}ATP, 0.5 M NaCl, in PEM_{100} buffer. Eluted proteins were centrifuged through a 5–20% sucrose gradient. Thirty-five 2-drop fractions were collected from the bottom of the tube. Duplicate 20 µl samples of sucrose gradient fractions 7–13 were subjected to SDS–PAGE and immunoblotted with HX-1 antibody. A 5 µl sample of the ATP/NaCl microtubule-eluted fraction was loaded into the final lane (labeled pre). All 35 gradient fractions were assayed for microtubule gliding; gliding activity is indicated below each fraction as described in Figure 3. NM, no motility.

extracted with 10 mM Mg^{2+}ATP and 0.5 M NaCl. Extraction with 10 mM Mg^{2+}ATP and 0.5 M NaCl was routinely used to obtain CENP-E-associated motor activity in subsequent purification steps. The remaining NaCl-resistant fraction likely represents the phosphorylation-sensitive, NaCl-resistant fraction of CENP-E (Liao *et al.*, 1994).

Sucrose density gradient sedimentation of the CENP-E-associated motor. Further purification of the CENP-E-associated motor was accomplished by fractionating the ATP/NaCl-eluted motor through a 5–20% sucrose gradient. An immunoblot of the gradient fractions (Figure 5) showed that CENP-E appeared in fractions 10–14, the identical fractions that exhibited motor activity. Coincident with the highest level of CENP-E, fraction 12 also contained the most gliding activity. A protein band having the same molecular mass as CENP-E was visible on a silver-stained SDS–PAGE gel of fractions 10–14, and the quantity of the protein correlated well with the amount of CENP-E that was detected by immunoblotting (data not shown). A second CENP-E cross-reactive protein band that migrated on SDS–PAGE gels a little faster than the major band could be detected on immunoblots of CENP-E-containing fractions when the protein was loaded directly onto the SDS–PAGE gel without prior immunoprecipitation (see lanes 12, 13 and 14 in Figure 5). The nature of the second CENP-E band is not known.

The gliding activity in each of the peak sucrose density fractions was characterized and found to be indistinguishable from the gliding activity previously described in D-100 fractions of HeLa mitotic extracts. Specifically, the gliding activity was minus-end directed and occurred at a mean rate of 0.22 ± 0.03 µm/s (25°C). In addition, the pattern of response to motor inhibitors (AMP-PNP, sodium orthovanadate, NEM and EHNA) and to GTP as a nucleotide substrate was identical to that reported in Figure 2 for the CENP-E-associated motor in D-100 fractions. Thus, the motor activity displayed the same rate, directionality and inhibitor specificity of the activity described in the less purified preparation.

CENP-E from the peak gel fractions was also immunoprecipitated with HX-1 to verify that depletion of CENP-E

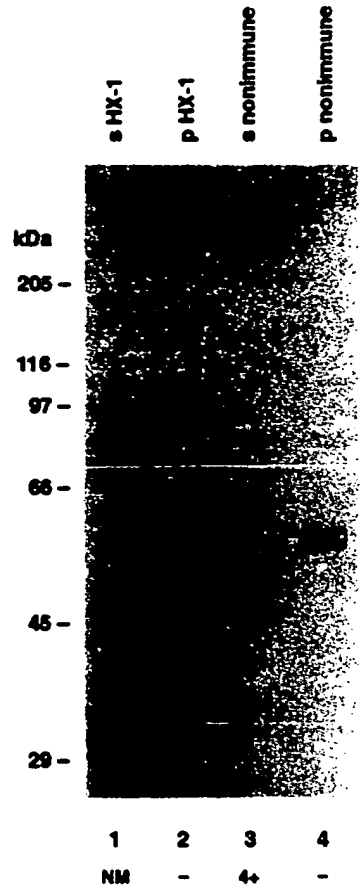

Fig. 6. Immunoprecipitation of sucrose gradient-purified CENP-E removed microtubule motor activity. Fractions 12 and 13 of the sucrose gradient (Figure 5) were pooled and immunoprecipitated with either HX-1 antibody or with non-immune serum. Pellet and supernatant fractions were subjected to SDS–PAGE and immunoblotted with HX-1. Supernatant after HX-1 immunoprecipitation (lane 1), pellet after HX-1 immunoprecipitation (lane 2), supernatant, non-immune serum control (lane 3), pellet, non-immune serum control (lane 4). Supernatants were assayed for motility as described in Figure 3. NM, no motility.

removed the motor activity. As shown in Figure 6, lanes 1 and 2, CENP-E was immunodepleted by HX-1 from pooled sucrose gradient fractions 12 and 13, and CENP-E appeared in the resulting pellet (p HX-1). The use of non-immune serum did not result in depletion of CENP-E (lanes 3 and 4). Immunodepletion of CENP-E resulted in the complete loss of microtubule gliding activity (Figure 6, lane 1).

Physical characteristics of the CENP-E-associated motor. The native molecular weight and axial ratio of the CENP-E-associated motor was estimated from sucrose density sedimentation and gel filtration data (data not shown). The motor activity co-migrated with CENP-E protein by gel filtration on Sepharose 4B 200, and a diffusion coefficient of 1.27 ± 0.04 × 10^7 cm^2/s was determined for the CENP-E-associated motor using standards of known diffusion coefficients (Bloom *et al.*, 1988). CENP-E-associated motor that had been extracted from microtubules with ATP/NaCl as described above was subjected to sucrose gradient sedimentation along with standards of known sedimentation coefficients (Materials

and methods). Three different determinations yielded a sedimentation coefficient for the CENP-E-associated motor of $12.6 \pm 0.7 \times 10^{-13}$ s. A native molecular weight for the CENP-E-associated motor of 874 000 ± 156 000 Da was calculated from the Svedberg equation assuming a partial specific volume of 0.725 ± 0.020 cm^3/g, a Stokes' radius of 16.9 ± 0.6 nm was calculated from the diffusion coefficient, and a frictional coefficient of 2.63 was derived from the molecular weight and Stokes' radius. We made the assumption that the overall shape of the CENP-E-associated motor, like kinesin (Bloom *et al.*, 1988), is approximated by a prolate ellipsoid, and calculated the axial ratio to be 40:1. Based on the foregoing parameters, the CENP-E-associated motor can be estimated to have dimensions of 2 nm × 80 nm.

Discussion

We have found that extracts of HeLa cells blocked in mitosis with vinblastine contain a minus-end directed microtubule motor activity that was not detected in extracts of interphase cells. Removal of CENP-E protein from the extracts with an antibody specific for CENP-E eliminated all microtubule gliding activity. CENP-E-associated motor activity was distinct from the activities of dynein and kinesin based upon microtubule gliding rates and nucleotide specificity and inhibitor specificity. Finally, the CENP-E motor activity co-purified with CENP-E protein in several purification steps.

Is the minus-end directed mitotic HeLa cell motor CENP-E?

Based on the evidence described in this work, we think that the minus-end directed motor present in mitotic HeLa extracts may be CENP-E itself. First, CENP-E is predicted to be a microtubule motor because a region near its N-terminus has a kinesin-like motor domain sequence that binds to microtubules in an ATP-sensitive manner (Yen *et al.*, 1992; Liao *et al.*, 1994). Second, the motor activity was removed from the mitotic D-100 fraction and from sucrose gradient fractions by immunoprecipitation with an antibody specific for CENP-E (Figures 3 and 6). Third, CENP-E protein co-fractionated with the motor activity in all purification steps, which included microtubule-affinity, gel filtration and sucrose density gradient centrifugation steps. Fourth, the motor activity was not due to dynein (also a minus-end directed motor) or to kinesin (Tables I and II). For example, blocking of dynein-mediated motor activity with a dynein-specific antibody had no effect on the CENP-E-associated motor activity (Figure 4). However, while it does not seem very likely, the present data do not formally exclude the possibility that the CENP-E-associated minus-end directed motor is due to an undetected protein that binds tightly to CENP-E. Such a protein might be a functional part of a CENP-E complex, or it could associate with CENP-E fortuitously.

Characterization of the CENP-E-associated motor

Rates of movement. The CENP-E-associated motor moved microtubules more slowly than kinesin, dynein or the KLP KIF3A, but somewhat faster than other KLPs such as Eg5, Kar3 and ncd. The CENP-E-associated motor moved microtubules at a mean rate of 0.46 μm/s at 37°C and 0.24 μm/s at 25°C, while the reported rates for microtubule movement with kinesin are 0.4–0.9 μm/s at 22–25°C (Vale *et al.*, 1985; Porter *et al.*, 1987; Saxton *et al.*, 1988) and the reported rates for HeLa cell dynein-mediated microtubule gliding are 4 μm/s at 37°C and 1.5 μm/s at 24°C (Lye *et al.*, 1989). (We obtained gliding rates of 0.9 μm/s with HeLa dynein at 25°C, see Figure 4A.) CENP-E-associated microtubule gliding was more rapid than gliding effected by the KLPs Eg5 (0.04 μm/s; Sawin *et al.*, 1992), ncd (0.07–0.12 μm/s; McDonald *et al.*, 1990; Walker *et al.*, 1990) and Kar3 (0.02–0.03 μm/s; Endow *et al.*, 1994), but slower than gliding effected by KIF3A (0.6 μm/s; Kondo *et al.*, 1994).

Direction of movement. In view of the fact that the motor domain of CENP-E is located at its N-terminus and that kinesin and other plus-end directed KLPs have their motor domains at the N-termini (Nislow *et al.*, 1992; Sawin *et al.*, 1992; Goldstein, 1993), it is curious that the CENP-E-associated motor isolated from prometaphase/metaphase HeLa cell extracts is minus-end directed (Figure 2). The KLPs that have been shown previously to be minus-end directed are ncd (McDonald *et al.*, 1990; Walker *et al.*, 1990) and Kar3 (Endow *et al.*, 1994), whose motor domains are at their C-termini. Such data have given rise to a model in which plus-end directed kinesin-like motors would have N-terminal motor domains and minus-end directed kinesin-like motors would have C-terminal motor domains. However, Stewart *et al.* (1993) found that chimeric proteins containing a C-terminal kinesin motor domain or an N-terminal ncd motor domain maintained their original directionality, demonstrating that the location of the motor domain in the primary protein sequence of ncd and kinesin does not determine the direction of movement. If CENP-E is responsible for the activity described here, it is the first naturally occurring minus end-directed motor whose motor domain is at its N-terminus.

Nucleotide and inhibitor specificities of the CENP-E-associated motor. The nucleotide and inhibitor specificities of the CENP-E-associated motor were distinct from other known motors. In particular, the nucleotide specificity for movement of CENP-E and kinesin were significantly different (Table I), indicating that kinesin-like motor domains do not necessarily have similar nucleotide specificities. Three out of four motor-inhibitory compounds likewise gave results that contrasted with those reported with dynein (Table II). The nucleotide specificity for the CENP-E-associated motor was also different from that of a Kar3 fusion protein (Endow *et al.*, 1994), KIF3A (Kondo *et al.*, 1994) and the kinetochore-associated motor activities described by Hyman and Mitchison (1991).

Physical characteristics of CENP-E and its associated motor activity. The native molecular weight of CENP-E was estimated to be ~874 000 Da as determined for bovine brain kinesin, assuming a partial specific volume for CENP-E of 0.725 ± 0.020 cm^3/g (Bloom *et al.*, 1988). Based upon its predicted amino acid sequence (Yen *et al.*, 1992) CENP-E itself would be expected to have a molecular weight of 312 000 Da. Other molecular motors including myosin, kinesin and dynein are complexes composed of one or two heavy chains and a number of

low-molecular-weight proteins. Similarly, CENP-E may be a dimer and/or it may complex with other proteins.

The calculated molecular dimensions of the native CENP-E-associated motor suggest that it is a long, rod-shaped structure (2 nm × 80 nm) which is twice the estimated length of kinesin (Bloom *et al.*, 1988). Because the amino acid sequence of the putative stalk domain of CENP-E is nearly four times longer than that of kinesin, it is possible that the putative stalk region is not as extended as the stalk region of kinesin. Alternatively, there are several interruptions of the predicted α-helical sequence of the stalk region of CENP-E (Yen *et al.*, 1992) which may create 'hinge regions' such as the bent region of the kinesin molecule which has been visualized by electron microscopy (Hisanaga *et al.*, 1989).

Possible mitotic functions of the CENP-E-associated motor. Microtubule motors are important for chromosome movements and spindle organization during mitosis (McIntosh, 1994). For example, mutations in the gene coding for ncd (Endow *et al.*, 1990) or for cytoplasmic dynein (Li *et al.*, 1993) yielded abnormal mitotic phenotypes. In addition, injection of antibodies to dynein or to the motor domain of kinesin blocked spindle pole separation in PtK_1 cells (Rodionov *et al.*, 1993; Vaisberg *et al.*, 1993) and injection of an antibody to the MKLP-1 motor prevented the PtK_1 cells from transitioning into anaphase (Nislow *et al.*, 1990). Also, microtubules attached to kinetochores of chromosomes *in vitro* can move relative to the kinetochores either in a plus-end directed or minus-end directed manner, indicating that kinetochores contain functional microtubule motors (Hyman and Mitchison, 1991).

The rate of rapid poleward movement of chromosomes that occurs soon after the capture of microtubules by kinetochores during prometaphase (Hayden *et al.*, 1990; Rieder and Alexander, 1990) has been reported to be 0.38 μm/s in PtK_1 cells at 37°C (Roos, 1976), and 0.13 μm/s 23–24°C (Merdes and De Mey, 1990). These rates are similar to the rates of microtubule gliding observed in the present study with the CENP-E-associated motor (0.46 μm/s at 37°C and 0.24 μm/s at 25°C). Because CENP-E is first detected at kinetochores at prometaphase, it is conceivable that CENP-E may participate in rapid poleward movement of chromosomes during prometaphase. In addition, because CENP-E remains at the kinetochores through metaphase, its motor activity may contribute to the oscillations (Skibbens and Skeen, 1993) of chromosomes as they congress toward the metaphase plate.

Materials and methods

Cell culture, S-100 extracts and D-100 fractions of mitotic and interphase cells

HeLa S3 cells (American Type Culture Collection) were grown in spinner cultures at 37°C in BioRich-1 medium (ICN, Irvine, CA) supplemented with modified Eagle's medium non-essential amino acids, penicillin (5000 U/ml), streptomycin (5 mg/ml), 10 mM HEPES buffer (Fisher, Los Angeles, CA), 1.5 g/l glucose and 2% calf serum. Mitotic cells were obtained by growing cells to a density of 4–6 × 10^5 cells/ml followed by incubation with 2–5 nM vinblastine sulfate for one cell cycle (22–24 h). Most (90–95%) of the cells were blocked in mitosis (Jordan *et al.*, 1991). Interphase cells were obtained from an exponentially growing cell culture; 96% of the cells were in interphase and 4% were in mitosis.

Cells were separated from the culture media by centrifugation. Cell pellets were washed by resuspension and sedimentation in phosphate-buffered saline and then in 50 mM piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid), 1 mM EGTA, 1 mM $MgSO_4$, 0.05% sodium azide (PEM_{50} buffer). Cell pellets were resuspended in equal volumes of PEM_{50} buffer containing 1 mM tosyl arginine methyl ester, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2.5 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride and 1 mM dithiothreitol (PEM_{50}/PID), and lysed by sonication at 0°C followed by incubation at 0°C for 20 min to ensure complete microtubule depolymerization. The lysate was centrifuged (100 000 *g*, 4°C, 30 min) and the resulting supernatant solution, which contained the HeLa mitotic motor activity, was called the S-100 extract. The S-100 extract could be stored frozen at −70°C for at least 4 months without loss of motor activity.

Fractionation of the S-100 extract by DEAE−cellulose (DE52, Whatman, Maidstone, UK) column chromatography yielded a soluble protein fraction (the D-100 fraction). The D-100 fraction was prepared by incubating the S-100 extract with an equal volume of DEAE−Sepharose for 40 min at 4°C, pouring the mixture into a 2.5 cm × 20 cm column, and eluting the column with PEM_{50}/PID buffer. The flow-through solution was concentrated to the volume of the original cell pellet with a Centriprep 30 centrifugal concentrator (Amicon, Beverly, MA) to form the D-100 fraction and frozen for later use in experiments or for further purification of mitotic motor activity.

Microtubule motor assays

A gliding assay in which microtubules move across the surface of glass coverslips (Vale *et al.*, 1985) was used to characterize the movement of the HeLa cell mitotic motor. Taxol-stabilized microtubules in 100 mM piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid), 1 mM EGTA, 1 mM $MgSO_4$, 0.05 % sodium azide (PEM_{100} buffer) and 10 μM taxol (National Cancer Institute) were diluted for use in motility assays to a tubulin concentration (in microtubules) of 0.1 μM. ATP or other nucleotides were added as appropriate. A 1 μl volume of protein fraction to be assayed for motor activity was placed on a 12 mm diameter glass coverslip and incubated at room temperature for 15–30 s. A 1 μl volume of the taxol-stabilized microtubule suspension was then added and the 12 mm coverslip was sandwiched between an 18 mm diameter coverslip and a pre-warmed microscope slide (Toso *et al.*, 1993). Microtubule images were captured by video-enhanced differential interference microscopy on a Zeiss IM-35 microscope with a temperature-controlled stage using a Hamamatsu C2400 (Newvicon) video camera (Toso *et al.*, 1993). Rates of microtubule movement were determined with Real Time Measurement software (generously provided by Dr N.Gliksman, Duke University Medical Center, NC and Dr E.Salmon, University of North Carolina) used with a Video Van Gogh image analysis card (Tekmatic Systems, Ben Lomond, CA). Gliding activity was scored as the reciprocal of the greatest dilution that supported directional microtubule movement as described in Porter *et al.* (1987).

The direction of microtubule gliding was determined by an axoneme gliding assay (Pryer *et al.*, 1986). Axonemes from *Strongylocentrotus purpuratus* and axoneme−microtubule constructs were prepared as described by Toso *et al.* (1993) except that a tubulin concentration of 10 μM was chosen to promote microtubule growth primarily at plus ends of axonemes. Tubulin was added to the gliding assay at a final concentration of 10 μM to prevent dilution-induced depolymerization of microtubules which could result in an incorrect assignment of polarity to axonemes. Some assays were carried out with axoneme−microtubule constructs formed with NEM-treated tubulin (Hyman *et al.*, 1991) mixed in equal proportion with untreated tubulin (final tubulin concentration, 20 μM) to prevent microtubule polymerization at minus ends of the axonemes.

Dynein antibody motility blocking assays (Vaisberg *et al.*, 1993) were performed by adsorbing dynein or D-100 fractions onto coverslips for 15 min and 30 s, respectively. Affinity-purified rabbit antiserum to *Dictyostelium* dynein (Bob) (a generous gift from Dr E.Vaisberg, University of Colorado, Boulder, CO) (0.6 mg/ml), or non-immune serum was added to coverslips and incubated for 15 min prior to the addition of microtubules and ATP.

Nucleotide specificity

Rates of microtubule movement were determined in the presence of 1 mM nucleotide; nucleotides were from Sigma, St Louis, MO. Commercially available analogs of ATP have been reported to be contaminated with as much as 1% ATP (Shimizu *et al.*, 1991). To control for the possibility that contaminating ATP might be responsible for observed microtubule motility with analogs, we incubated the ATP analogs with an ATP-depleting solution (see below) and repeated the gliding assays. With dideoxy ATP and the deoxy derivatives of ATP, the rates of microtubule

movement were unchanged by ATP depletion. However, a very slow rate of movement that had occurred initially with 8-azido ATP and with ATPγS was eliminated by depletion of ATP, indicating the presence of contaminating ATP in these two analog preparations.

Inactivation of dynein by the vanadate–UV photocleavage reaction

Samples of HeLa cell dynein (purified by the method of Pfarr *et al.*, 1990) or D-100 fractions were illuminated with a 365 nm light source at a distance of 2.5 cm in the presence of 10 μM sodium orthovanadate (Fisher) at 0°C for 50 min (Gibbons *et al.*, 1987). Samples were then incubated with a solution of 5 mM norepinephrine (Sigma) for 20 min at 0°C to inactivate the vanadate which otherwise would inhibit motility of dynein and the CENP-E-associated motor. Control samples were illuminated with UV light in the absence of vanadate. Treatment of control samples with norepinephrine did not affect gliding rates.

Antibody purification, immunoprecipitations and immunoblots

The generation and characterization of the antibody HX-1, a rabbit polyclonal antiserum raised to a fusion protein of a bacterially expressed segment of the putative CENP-E stalk region and which identifies a protein of size consistent with the predicted size of CENP-E on immunoblots of chromosomal proteins, is described in Yen *et al.* (1992), where it is referred to as pAb1. Antibody HX-1 was partially purified by ammonium sulfate fractionation and DEAE-adsorption prior to use in immunoprecipitation reactions (Harlow and Lane, 1988). Immunoprecipitation was performed by incubating a 1:200 dilution of the purified antisera with the sample (1 h, 0°C), followed by a similar incubation with an equal volume of *Staphylococcus* protein A–Sepharose and centrifugation to sediment the immunoprecipitated proteins. The supernatants were saved and the pellets washed extensively in PEM_{50} buffer to remove non-specifically bound proteins. Pellets were resuspended in an equal volume of SDS–PAGE sample buffer. Pellets and supernatants were subjected to SDS–PAGE (Laemmli, 1970). Gels were immunoblotted as described previously (Thrower *et al.*, 1991), with the following modifications; electrotransfers for CENP-E and dynein were carried out at 0.5 A for 2 h, HX-1 serum was used at a 1:500 dilution, and affinity-purified rabbit antiserum to *Dictyostelium* dynein (Bob) was used at 0.4 μg/ml.

Microtubule-affinity purification of the CENP-E-associated motor

A 40 ml sample of packed HeLa cells was processed as described above to obtain the D-100 fraction which was depleted of ATP by incubation with hexokinase (10 U/ml) and 50 mM glucose at 20°C for 30 min followed by centrifugation (100 000 *g*, 30 min, 10°C) to remove actomyosin. The pellet was discarded and taxol was added to the supernatant to a final concentration of 10 μM. Two-hundred μmoles of microtubule-associated protein-depleted bovine brain tubulin (Toso *et al.*, 1993) was polymerized in a separate tube by the addition of an equimolar concentration of taxol. The taxol-stabilized microtubules were then added to the supernatant from the previous step and incubated for 30 min at 4°C to allow HeLa cell proteins to bind to the microtubules. The microtubules were sedimented (4°C, 30 min, 100 000 *g*) through a 10 ml 20% sucrose cushion in PEM_{50}/PID buffer. The microtubules with adhering proteins were then resuspended in PEM_{100}/PID buffer containing 10 μM taxol and centrifuged (4°C, 10 min, 100 000 *g*, Beckman SW 50.1 rotor). Microtubule-binding proteins were dissociated from the microtubules by incubation with 400 μl of 10 mM $MgSO_4$, 10 mM ATP and 0.5 M NaCl, in PEM_{100}/PID buffer containing 10 μM taxol (1 h, 4°C) with centrifugation as in the previous step. Microtubule-binding proteins were further fractionated by centrifugation (14 h, 4°C, 200 000 *g*) through a 4 ml 5–20% sucrose gradient in PEM_{100} buffer.

Molecular weight and dimensions of the CENP-E-associated motor

The sedimentation coefficient of the CENP-E-associated motor was determined by sucrose density centrifugation with protein from three independent purifications. Protein standards were: cytochrome *c* (1.71S), bovine serum albumin (4.73S), rabbit IgG (7.05S), catalase (11.3S) and thyroglobulin (19.4S) (Sober, 1970). Assignment of a sedimentation coefficient for CENP-E was accomplished by plotting sedimentation coefficients of the standards as a function of their elution positions and comparing the elution position of CENP-E with the standards by linear regression analysis.

The diffusion coefficient, $D_{20,w}$, of CENP-E was measured by gel filtration with a 20 ml Sepharose 4B-200 (Pharmacia, Piscataway, NJ) column equilibrated in PEM_{100} and 10% (w/v) glycerol. Protein standards and their diffusion coefficients were: myosin (1.16), bovine lens α crystallin (2.00), *E.coli* β galactosidase (3.12), *Bacillus* urease (3.46), and β amylase (5.77) ($\times 10^7$ cm^2/s) (Sober, 1970). K_{av} values were calculated from the formula $K_{av} = (V_e - V_0)/(V_t - V_0)$. Mean K_{av} values of standards were plotted versus $1/D_{20,w}$ and the diffusion coefficient of CENP-E was calculated by linear regression analysis.

The native molecular weight, Stokes' radius, and axial ratio of CENP-E were derived from the sedimentation coefficient and diffusion coefficient as described for kinesin by Bloom *et al.* (1988). The partial specific volume, $\bar{v}$, was assumed to be 0.725 ± 0.020 cm^3/g (the mean of 13 globular and fibrous proteins commonly used as molecular weight standards; Tanford, 1961). The Stokes' radius was calculated from the diffusion coefficient (Bloom *et al.*, 1988). The axial ratio was derived from Table 12.1 of Freifelder (1982), using a frictional ratio, $f/f_0 = a \div (3\bar{v}\ M_r/4\pi N)^{1/3}$ with *a* equal to Stokes' radius and N equal to Avogadro's number (Siegel and Monty, 1966).

Acknowledgements

We thank Mr Herb Miller for the preparation of tubulin and Drs John Carbon and Kim Middleton for critical reading of the manuscript. These studies were supported by American Cancer Society Grant DHP-43F. T.J.Y. is a Lucille C.Markey Scholar supported by USPHS GM44762, CA06927 and the Markey Charitable Trust.

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Received on September 30, 1994; revised on December 8, 1994